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(54) Title: HUMAN FACILITATIVE GLUCOSE TRANSPORT PROTEIN GLUT8			
(57) Abstract			
<p>The nucleotide sequence encoding a novel facilitative glucose transport protein GLUT8 was determined and a protein sequence was deduced. Detection of expression of the protein is useful as a diagnostic and staging marker in cancer, espacially breast cancer. Control of expression of the protein can be useful in cancer therapy. Similar to GLUT4, upregulation of the protein is useful in treating non-insulin dependent diabetes mellitus (NIDDM).</p>			

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HUMAN FACILITATIVE GLUCOSE TRANSPORT PROTEIN GLUT8

The present invention relates to a novel glucose transporter protein isolated from a breast cancer cell line, and to the gene encoding the protein. Detection of
5 expression of the protein is useful as a diagnostic and staging marker in cancer, especially breast cancer. Control of expression of the protein is useful in the therapy of cancer. Upregulation of the protein is useful to overcome insulin resistance in non-insulin dependent
10 diabetes mellitus.

BACKGROUND OF THE INVENTION

Transport of blood glucose across the plasma membrane occurs via facilitative glucose transport,
15 catalysed by a family of facilitative-diffusion glucose transporter molecules. The mammalian glucose transporters (GLUTs) are a group of closely-related facilitative hexose transporter proteins which are expressed in a tissue-specific manner. The pattern of expression reflects both
20 the kinetic and substrate binding characteristics of the transporters and the sugar requirements of individual tissues. In human tissues, five members of the facilitative glucose transporter family have been identified. The cDNAs for GLUTs 1, 2, 3, 4 and 5 have been
25 cloned and sequenced, their tissue distribution determined and their kinetic properties studied. GLUT6 had been designated as a further member of this family, but was subsequently shown to be a pseudogene. In rat liver, an isoform with strong sequence similarity to GLUT2 and
30 designated as GLUT7 has been reported. However, no evidence that this transporter is expressed in humans has been shown (reviewed by Bell et al 1993).

Expression of the GLUT4 isoform is restricted to skeletal muscle and adipose tissue. Under basal conditions
35 most GLUT4 protein resides in intracellular compartments. When muscle cells and adipose tissue are exposed to insulin, GLUT4 protein is translocated to the plasma

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membrane, where it is able to transport glucose into cells. The complex mechanisms which are involved in this process of translocation to the plasma membrane in response to insulin are still being elucidated. It is known that phosphorylation of specific amino acids controls trafficking of GLUT4. Extensive studies have concluded that mutations in the GLUT4 gene are not present in patients with non-insulin dependent diabetes mellitus (NIDDM) (Kahn, 1994). However, it is thought that defects in the insulin-regulated translocation of GLUT4 may play a part in insulin resistance associated with NIDDM.

Malignant cells are rapidly dividing, and therefore have increased glucose requirements. Oxidative metabolism is generally impaired in these cells, and tumour cells are characterised by high rates of glucose uptake, lactate formation and glycolysis. The levels of expression of both GLUT1 (erythrocyte/HepG2 glucose transporter) and GLUT3 (brain/foetal glucose transporter) isoforms can be elevated in malignant cells. For example, mRNA levels of GLUT1 and GLUT3 are significantly elevated in oesophageal, stomach and colon cancers and primary brain tumours. There is much evidence to indicate that upregulation of glucose transport is a fundamental part of the malignant process.

As in other tumours, malignancy in breast tumours is associated with altered metabolism and increased glucose uptake. In normal mammary epithelial cells only the GLUT1 isoform is expressed, and levels of transporter are altered by hormonal influences during lactation and weaning. Some breast tumours over-express GLUT1. The presence of GLUT4, GLUT2 and GLUT5 in breast tumour cells has also been reported.

Our work originated from the hypothesis that the hormonal factors, such as oestrogen, which influence breast cancer progression and cell proliferation might be involved in regulation of heterogeneous glucose transporter expression in breast cancer. We have now surprisingly found that a new type of glucose transporter protein, which

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is related to but distinct from GLUT4, is present in a malignant breast epithelial cell line, and that this transporter protein is also present in human skeletal muscle and adipose tissue cells.

5

SUMMARY OF THE INVENTION

According to a first aspect, the invention provides a nucleic acid molecule encoding a novel facilitative glucose transporter protein which we have
10 designated GLUT8. The nucleic acid sequence may be genomic DNA, cDNA, or RNA, and may be single stranded or double stranded. Preferably, the nucleic acid is cDNA. More preferably, the nucleic acid molecule has the sequence as set out in SEQ ID NO:4. Although the invention is
15 described in detail in relation to cDNA, the person skilled in the art will be able to utilise known methods in order to prepare nucleic acid sequences of other kinds.

The person skilled in the art would also appreciate that the present invention provides a nucleic
20 acid molecule or fragment thereof which hybridizes under stringent conditions to the sequence set out in SEQ ID NO:4. "Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% NaDodSO₄
25 (SDS) at 50°C, or (2) employ during hybridisation a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at
30 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at
35 42°C in 0.2 x SSC and 0.1% SDS.

In a second aspect, the invention provides the transporter protein GLUT8, and biologically-active

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fragments, analogues and derivatives thereof. Preferably, the transporter protein has greater than 70% sequence homology with the amino acid sequence set out in SEQ ID NO:5. More preferably, greater than 80% sequence homology.

5 Even more preferably, greater than 95%, and most preferably, the protein has the amino acid sequence as set out in SEQ ID NO:5. Using methods known in the art, the skilled person will be able to identify biologically-active fragments or analogues of the protein described in detail
10 herein. Methods such as site-directed mutagenesis may be used to prepare nucleic acid sequences encoding substitutions, deletions and additions to the naturally-occurring gene and amino acid sequences.

In a third aspect, the invention provides a
15 method of diagnosis of a malignant condition, comprising the step of detecting expression or activity of GLUT8 in a tissue or cell.

In a fourth aspect, the invention provides a method of monitoring of efficacy of treatment of a
20 malignant condition, comprising the step of detecting activity or expression of GLUT8 in a tissue or cell.

According to a fifth aspect, the invention provides a method of selecting a method of treatment of a malignant condition, comprising the step of measuring the
25 ability of a proposed therapeutic agent to inhibit activity or expression of GLUT8 in a tissue or cell.

It is contemplated that non-utilisable glucose analogues targeted to the malignant tissue will be particularly suitable for inhibiting expression and/or
30 activity of GLUT8 for treatment of cancer.

It is also contemplated that anti-sense nucleic acid sequences directed against the GLUT8 nucleic acid sequence will be useful for inhibiting expression of GLUT8.

It will be further contemplated that
35 dominant/negative mutants of GLUT8 nucleic acids or protein which retains some function will be useful for inhibiting the growth of breast cancer.

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Preferably the malignant condition is breast cancer, but it is also contemplated that methods of the invention will be useful for treatment of prostate cancer and other epithelial cell cancers, particularly skin
5 cancers, including malignant melanoma, and colon cancers.

In a sixth aspect the invention provides an antibody directed against GLUT8. The antibody may be polyclonal or monoclonal, but is preferably polyclonal. Preferably, the antibody is directed against the C-terminal
10 region of GLUT4. More preferably, the antibody is directed against one or more epitopes present in the sequence NKLCGRGQSRQLSPET (SEQ ID NO:12).

Methods for production and screening of monoclonal antibodies are very well known in the art. The
15 antibodies of the invention are useful for assay of GLUT8 protein, for example by radioimmunoassay, ELISA assay, and by immunocytochemical detection. Some antibodies of the invention have the ability to inhibit the activity of GLUT8, and the person skilled in the art will readily be
20 able to identify whether or not a given antibody has such inhibitory activity. It will be clearly understood that fragments such as Fv, Fab and F(ab)₂ and analogues such as ScFv and humanised antibodies which are able to bind to and/or inhibit GLUT8 are within the scope of the invention.
25 Again methods for production of such fragments and analogues are well known in the art. See for example, Australian Patent No 690528, International Patent Application No PCT/AU93/00491 and No PCT/AU98/00212, and references cited therein.

30 Because of its homology with GLUT4, and because of the known involvement of GLUT4 and the known effect of insulin on translocation of GLUT4 to the plasma membrane, it is contemplated that upregulation of GLUT8 expression will be useful to overcome insulin resistance in non-
35 insulin dependent diabetes mellitus.

Thus in a seventh aspect the invention provides a method of treatment of non-insulin dependent diabetes

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mellitus, comprising the step of upregulating expression of GLUT8 in a tissue or cell. Preferably the tissue is skeletal muscle and/or adipose tissue. For example tissue-localised gene therapy may be used for expression of GLUT8
5 in skeletal muscle in order to stimulate glucose uptake.

It will be appreciated by the skilled person that mutations in the GLUT8 gene or regulatory sequences may be involved in NIDDM. As such, genetic mutation of GLUT8 may have a causal or exacerbating effect with regards to NIDDM.
10 Thus, the person skilled in the art would appreciate that mutations in the GLUT8 gene or regulatory regions may be corrected by gene therapy.

Accordingly, in an eighth aspect the invention provides a method of detecting a mutation in the GLUT8 gene
15 or regulatory sequence of a patient comprising the step of analysing the gene or regulatory sequence for a nucleic acid change compared to that set out in SEQ ID NO: 3 or 4. Preferably, the patient is a NIDDM patient, and the method of detection is single stranded conformational polymorphism
20 (SSCP) or other genetic analysis procedure known in the art.

In a ninth aspect the invention provides a method of screening putative agents for treatment of cancer, comprising the step of measuring the ability of the agents
25 to inhibit the activity of GLUT8 *in vitro* or *in vivo*.

In a tenth aspect the invention provides a method of screening putative agents for treatment of diabetes and/or insulin-resistance syndrome comprising the step of measuring the ability of the agents to upregulate or
30 enhance the activity of GLUT8 *in vitro* or *in vivo*.

In the third, fourth and fifth aspects of the invention, expression of GLUT8 may be detected by a variety of different means, including but not limited to immunocytochemistry, hybridisation analysis, PCR, RT-PCR
35 and the like, using a sample of tissue or of biological fluid suspected to contain cancer cells.

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Activity of GLUT8 *in vivo* may for example be detected by positron emission tomography scanning using a hexose labelled with a fluorescent marker; preferably the hexose is a glucose analogue or hexose specifically transported by GLUT8.

It will be clearly understood that for the purposes of this specification the word "comprising" is to be understood to mean "including, but not limited to".

Unless specifically described herein, methods utilized are generally known in the art, for example, by reference to Sambrook et al (1989).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1a shows the results of Western blotting using proteins extracted from the malignant breast epithelial cell lines MCF7 and T47-D, and GLUT4 C-terminal polyclonal antibody (R820; James et al., 1989).

Figure 1b shows the results of Northern analysis of RNA extracted from MCF7 malignant breast epithelial cells probed with GLUT4 cDNA.

Figure 2 shows the results of Southern hybridisation analysis using GLUT4 cDNA to probe RT-PCR products isolated from MCF7 cells and T47-D cells. The primers used were the degenerate primer SEQ ID NO:3 and reverse primer, SEQ ID NO:2. GLUT4 cDNA was used as positive control.

Figure 3 shows the initial sequence alignment of the deduced amino acid sequence corresponding to the 350 bp PCR fragment with human GLUT1, GLUT2, GLUT3, GLUT4, GLUT5 and rat GLUT7.

Figure 4 shows genomic DNA extracted from MCF7 cells and whole blood, digested with restriction enzymes *Pst* 1, *EcoR* 1, separated on agarose gels and transferred to nylon membrane by Southern transfer. Filters were probed with the GLUT8 full-length cDNA probe.

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Figure 5 shows PCR amplified products obtained using the GLUT8 specific primer pair 1 on genomic DNA from MCF7 cells. Southern blot was probed with GLUT8 cDNA.

Figure 6 shows the sequence alignment between
5 part of the deduced amino acid sequence of the first GLUT8 cDNA clone and the sequences of human GLUT1, GLUT2, GLUT3, GLUT4, GLUT5.

Figure 7 illustrates amino acid sequence
homologies between different GLUT8 regions and
10 corresponding regions of human GLUT1 to GLUT5, and with the binding sites of other facilitative glucose transporters:

- a) The putative substrate binding site in helix 7;
- b) The cytochalasin B binding site;
- 15 c) Binding sites of other glucose transporters:
ARA: *E. coli* AraE arabinose transporter
YHT: Yeast SNF3 glucose transporter
MST1: monosaccharide transport protein from
Nicotiana tabacum.

20 Figure 8 shows the results of RT-PCR detection of GLUT8 in human tissues and cell lines.

Figure 9 shows the results of Southern analysis
of RT-PCR using primer pair 1, indicating preferential
expression of GLUT8 in malignant breast tissue compared to
25 normal tissue.

- a) Breast tumour sample, normal breast tissue, and MCF7 cells, probed with GLUT8 (primer pair 2)
- b) GLUT1 to GLUT5, GLUT8, negative control, and GLUT1 cDNA (positive control), probed with GLUT1 (primer
30 pair 1)
- c) GLUT1 to GLUT5, GLUT8, negative control and GLUT4 plasmid (positive control) probed with GLUT4 (primer pair 1).

Figure 10 shows the results of immunocytochemical
35 detection of GLUT8 in cultured malignant breast cells and in normal and malignant breast tissue

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- a) Breast tumour sample incubated with non-immune serum
- b) Breast tumour sample incubated with immune serum
- 5 c) Normal breast tissue incubated with non-immune serum.

Figure 11 shows immunocytochemical detection of GLUT8 in MCF7 cells under basal conditions, and following insulin treatment for 15 min. GLUT8 antiserum is used at 10 1/100 and 1/300 dilutions. Specific staining is competed by competitive, but not non-competitive peptide.

Figure 12 shows a Western Blot of a 50 kDa protein species detected by GLUT8 antisera in MCF7 and T47-D cells by affinity purified antiserum (AP) in MCF7 cells.

15 Figure 13 shows MCF7 protein extracts following membrane fractionation and deglycosylation.

Figure 14 demonstrates that the GLUT4 monoclonal antibody, 1F8 does not detect a protein of 50 kDa in MCF7 cells. Rat gastrocnemius muscle protein extracts are used 20 as a positive control for GLUT4 protein.

Figure 15 shows GLUT8 protein being detected in both the rat adipose tissue and skeletal muscle by GLUT8 antiserum.

Figure 16a shows the detection of GLUT8 protein 25 in human adipose tissue and skeletal muscle.

Figure 16b Immunohistochemical detection of GLUT8 in human skeletal muscle.

Figure 17 shows *in vitro* transcription/translation of GLUT4 and GLUT8 mRNA in the presence and 30 absence of microsomes.

Figure 18 shows the effects of preincubation with insulin on immunocytochemical staining of MCF7 breast tumour cells:

- a) Pre-immune serum
- 35 b) Immune serum without insulin pretreatment
- c) Immune serum after preincubation with 10 nM insulin for 15 min.

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Figure 19 shows immunofluorescence staining of GLUT8 in MCF7 cells under basal conditions, and after long-term exposure to insulin.

Figure 20 shows a Northern blot analysis of polyA RNA extracted from MCF7 cells. Specific transcripts of approximately 4.4 and 2.5 kb were detected with GLUT8 cDNA.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described by way of reference only to the following non-limiting examples and to the figures.

Abbreviations used herein are as follows:

PCR	Polymerase Chain Reaction
15 RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
GLUT	Glucose Transport Protein
Mab	Monoclonal antibody
SSPE	Sodium Chloride, Sodium Orthophosphate and EDTA buffer
20 SDS	Sodium Dodecyl Sulphate

Example 1 Identification of a GLUT4-Related Sequence in a Breast Cancer Cell Line

Expression of GLUT4 is normally tightly restricted to skeletal muscle and adipose tissue. Using standard methods we detected a protein of similar molecular weight (49 kd) to GLUT4 in two malignant breast epithelial cell lines, MCF7 and T47-D Soule et al. 1973; Keydar et al 1979, respectively). Briefly, total cell homogenates (1.5 µg/µl) were subjected to SDS-polyacrylamide electrophoresis. Proteins were transferred to membranes and after blocking for non-specific binding were incubated with GLUT4 polyclonal anti-sera diluted 1:300 (James et al. 1989). Immunoreactive proteins were detected using ¹²⁵I-labelled Protein A, and the results are shown in Figure 1a. Rat jejunum and gastrocnemius muscle, bovine aortic endothelial cells (BAEC), L6 rat myoblast cells, and 106.01

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rat malignant bone cells were used for comparison. A GLUT4 like protein was detected in the two malignant breast cell lines and in the skeletal muscle sample, but not in other tissues or cells.

5 Northern analysis showed a specific transcript of 2.8 kb when RNA extracted from MCF7 cells was probed with the GLUT4 cDNA (cDNA probe provided by D.E. James). Rat liver and gastrocnemius muscle RNA samples were used as negative and positive controls respectively. The results
10 are summarised in Figure 1b. This transcript was smaller than would be expected for GLUT4 (2.8 kb compared to 3.5 kb) for a human transcript.

Example 2 The Protein is Distinct from GLUT4

15 To confirm the presence of GLUT4 in MCF7 cells, we designed primers based on the GLUT4 sequence and performed RT-PCR reactions on RNA extracted from MCF7 and T47-D cells. Total RNA was extracted using Trizol reagent (Life Technologies), according to the manufacturer's
20 instructions. Reverse transcriptase reactions were performed with Reverse Transcriptase enzyme and oligo dT primers (Promega) using conditions recommended by the manufacturer. GLUT4 primers were as follows:

25 Forward primer:

TTTGAGATTGGCCCTGGCCCAT

SEQ ID NO:1

Reverse primer:

GTC (AG) TTCTCATCTGGCCCTAA

SEQ ID NO:2

30 PCR was performed at an annealing temperature of 49°C and 30 sec extension for 40 cycles, using Taq DNA polymerase purchased from Boehringer. We were unable to confirm the presence of GLUT4 by this method.

35 Example 3 Isolation and Sequencing of a PCR Fragment

On the assumption that the protein detected by Western and Northern blots might therefore be a GLUT4-like

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protein, we performed further PCR reactions. Using reduced annealing temperatures and degenerate primers, a PCR product was obtained. The PCR primers were designed to encompass the region from the transmembrane domain 10 (TM10) to the C-terminus of a proposed facilitative glucose transporter. All members of the GLUT family have a high degree of homology of TM10. It was expected that a GLUT4-like protein would possess some similarity to GLUT4 in the C-terminal region, particularly as the GLUT4 polyclonal antibody which we used for the experiments in Example 1 was designed to interact with the C-terminus. Primers used in these experiments were:

Forward primer:

15 TTTGAGATTGGNCC(TAC)GGCCC(CG)AT SEQ ID NO:3

and reverse primer of SEQ ID NO:2 as defined above. The PCR reaction conditions utilised touchdown PCR, with the first 5 cycles at an annealing temperature of 37°C followed by 35 cycles at an annealing temperature of 49°C. The extension time was 30 sec. For these experiments, the Expand High Fidelity PCR System (Boehringer) was used. A 350 bp PCR product was obtained. This PCR product hybridised to the GLUT4 cDNA by Southern analysis, as shown in Figure 2.

The 350 bp PCR fragment was purified from agarose gels and sequenced by direct incorporation using the fmol DNA Sequencing System (Promega), and determined to be distinct from nucleic acid encoding GLUT4. Approximately 250 bp of nucleotide sequence were obtained, and Figure 3 shows initial sequence alignments obtained using the deduced amino acid sequence. This showed that the new protein, designated GLUT8, had only 60% homology to human GLUT4.

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Example 4 Isolation of a Full-Length Clone Encoding
GLUT8

Searches of data bases allowed us to obtain an expressed sequence tag (EST) clone which encompassed the sequence which we had obtained by PCR from MCF7 cells. The clone (I.M.A.G.E. Consortium Clone ID 43006), was purchased from Genome Systems, Inc., St. Louis, USA. This clone is 1.2 kb, and includes 500 bp of coding region and a further 700 bp of 3' UTR sequence. The EST clone was isolated from a neonatal human brain library.

The EST clone enabled us to perform library screens in order to obtain a full-length clone. We were unable to obtain any positive cDNA clones from a λ Zap cDNA library prepared from malignant breast epithelial cells (provided by R Sutherland, Garvan Institute, Sydney, Australia). Approximately 2×10^6 plaques were screened under high stringency conditions. Hybridisation was performed at 42°C, 6 x SSPE washes

- 1) 15 mins at 42°C; 2 x SSPE,
 - 2) 30 mins at 65°C; 1 x SSPE,
 - 3) 15 mins at room temperature; 0.1 x SSPE,
- All wash solutions contained 0.1% SDS.

All of the putative positive clones obtained proved when sequenced to be fusions of the GLUT1 isoform, which presumably arose as a result of chromosomal rearrangements in these tumour cells.

A whole human embryo cDNA λ gt10 library was then screened. 5×10^5 plaques were screened under high stringency hybridisation conditions. Our rationale for choosing this library was based on the similar glucose requirements of rapidly-dividing foetal and tumour cells, and the fact that the EST clone was isolated from a neonatal library. Two positive cDNAs, one of which was full-length, were isolated from the λ gt10 library.

The full-length cDNA sequence is shown in SEQ ID NO:4. It proved extremely difficult to clone these cDNAs into plasmid vectors. Our subsequent data suggest that the

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most likely reason for this difficulty is the close homology (and hence subsequent homologous recombination) with *E. coli* sugar transporter molecules. These cloning problems were eventually overcome by rigorous use of recombination-deficient *E. coli* strain (Sure 2 cells, Stratagene; Hatt et al, 1992). The stability of the cDNA clones in plasmid vectors appears to be orientation-dependent.

10 Example 5 GLUT8 Genomic DNA

Genomic DNA was extracted from MCF7 cells and whole blood from three human subjects (2 female and 1 male). DNA (20 µg) was digested with restriction enzymes *Pst* 1, *EcoR* 1, *BamH* 1 or *Hind* 111, separated on agarose
15 gels and transferred to nylon membrane by Southern transfer. Filters were probed with the GLUT8 full-length cDNA probe. Similar restriction patterns were obtained from MCF7 and normal human DNA samples confirming that the GLUT8 gene is present in the normal human genome. The size
20 of the GLUT8 gene is estimated at 15-18kb. These results are illustrated in Figure 4.

PCR reactions were performed on genomic DNA from MCF7 cells using the GLUT8 specific primer pair 1, as described in Example 7. The PCR product obtained was
25 transferred to nylon membrane, and Southern blots probed with cDNAs for GLUT1, 2, 3, 4, 5 and 8. Only the GLUT8 probe hybridised to the PCR amplification product obtained with Primer pair 1 as is shown in Figure 5. These results confirm that the primers used are specific for a gene which
30 is unique from GLUT1, 2, 3, 4 and 5.

Example 6 Sequence Analysis

The deduced amino acid sequence of the GLUT8 isolated from the embryonic library is set out in SEQ ID
35 NO:5.

This shows considerable homology to the facilitative glucose transporter family, as shown in

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Figures 6 and 7. There is homology between proteins from human, bacterium, yeast and plant. The trans-membrane domains, substrate binding site and cytochalasin B binding site are highly conserved. A leucine-leucine motif is present in the C-terminus. This motif is known to be involved in the control of GLUT4 trafficking (Verhey and Birnbaum, 1994). At the nucleotide level, the sequence of the isolate is most closely related to those of GLUT4 and the *E. coli* xylose and arabinose transporters. There is also considerable homology to yeast hexose transporter proteins.

Example 7 Detection of GLUT8 in Normal and Malignant Tissue

The messenger RNA for GLUT8 has not been detected by Northern blot analysis in any normal adult rat tissue which we have examined so far. Fresh tissues were frozen in liquid nitrogen and total RNA extracted by the method of Chomcynski and Sacchi (1987). The tissues tested were liver, brain, intestine, kidney, testis, heart, skeletal muscle, adipose tissue, and spleen. RNA was separated by denaturing agarose gel electrophoresis, transferred to nylon membrane (Amersham), and hybridised according to the manufacturer's instructions using the GLUT8 cDNA as a probe. In addition GLUT8 was not detected by Northern analysis in RNA from foetal brain, lung, kidney or liver. For these experiments, a foetal human Northern blot (Clontech) was probed with the GLUT8 cDNA.

Using primers which are specific for GLUT8, we have detected the presence of GLUT8 by RT-PCR in a human prostate cancer cell line, PC-2, and in human skeletal muscle, skin and to a lesser extent in adipose tissue. Primers and PCR conditions were as described below. The results are shown in Figure 8. A Chinese hamster ovary cell line gave a negative result.

We have performed semi-quantitative RT-PCR analysis of RNA extracted from 10 human breast cancer

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samples and from morphologically normal breast tissue taken from the same patients. Samples of tissue were collected at surgery and frozen in liquid nitrogen.

RNA extraction and reverse transcription were performed as described in Example 1.

Primers were designed on the basis of the GLUT8 sequence. PCR was performed with Taq polymerase (Boehringer) using manufacturer's buffer and recommendations. Two primer pairs were used:

10 Primer Pair 1

Forward primer:

TCCATGGCTGGAAGTACAT

SEQ ID NO:6

15 Reverse primer:

TAAGTGTTCTGGCACTATC

SEQ ID NO:7

Primer pair 1 was used in a PCR reaction, the conditions of which were annealing temperature of 50°C and extension for
20 1 min for 40 cycles.

Primer Pair 2

Forward primer:

25 TCAACATCCACATGAACT

SEQ ID NO:8

Reverse primer:

TGAAAAAGCAGCAACATAAAC

SEQ ID NO:9

30 Primer pair 2 was used in a PCR reaction, the conditions of which were an annealing temperature of 53°C and a 30 sec extension for 40 cycles.

These primers are specific for GLUT8, and do not amplify DNA from cDNA constructs of GLUTs 1, 2, 3, 4 or 5.

35 The GLUT1 cDNA probe was obtained from D.E. James (University of Queensland, Australia) and GLUT2, 3 and 5 cDNA probes from G. Bell (University of Chicago, USA).

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Amplified PCR products were examined by agarose gel electrophoresis and Southern blots probed with the cDNAs for GLUT4 and GLUT8. The products hybridised only to GLUT8. Normalisation of RNA concentration, RT-PCR reactions and DNA loading were performed by amplification of a non-oestrogen-dependent house-keeping gene-36B4 (Labora, 1991). Specific primers for the 36B4 cDNA were synthesised. The PCR reaction was performed with an annealing temperature of 65°C and extension of 30 secs for 20 cycles.

Forward primer:

TGGGCTCCAAGCAGATGC

SEQ ID NO:10

Reverse primer:

GGCTTCGCTGGCTCCCAC

SEQ ID NO:11.

Our preliminary results indicate a higher level of expression of GLUT8 in the tumour tissue compared to normal breast tissue, with GLUT8 being undetectable in some normal samples. This is illustrated in Figure 9.

Example 8 Production of Antibody Directed to the C-Terminal Region of GLUT8

We have synthesised a peptide based on the terminal 16 amino acids of the sequence of the C-terminus of GLUT8, as determined from the nucleotide sequence. The C-terminal region has been successfully targeted to produce polyclonal antibodies for GLUTs 1 and 4. A 16 mer peptide

NKLCGRGQSRQLSPET

SEQ ID NO:12

was synthesised, and 2 mg was coupled to 6 mg of N-succinimyl-3-[2-pyridyldithio]propionate (SPDP) activated keyhole limpet haemocyanin (KLM) through the internal

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cysteine residue according to the manufacturer's instructions (Pierce Chemical Company). Three rabbits were immunised subcutaneously with peptide conjugate (500 µg) emulsified in Freund's complete adjuvant, and boosted at 5 2 week intervals with peptide conjugate (500 µg) emulsified in Freund's incomplete adjuvant. Final bleeds were taken after the third boost. Anti-serum at a dilution of 1:300 was used for immunocytochemical detection of GLUT8, using the immunoperoxidase reaction in cultured malignant breast 10 cells. Specificity of detection of GLUT8 protein was confirmed by using serial dilutions and comparison to sera from pre-immune bleeds. The results of immunocytochemical detection are illustrated in Figure 11, which shows that the immune serum at a dilution of 1:100 gave strong 15 staining of MCF7 cells. Serum from a pre-immunization bleed gave a negative result. The immune serum is designated R1396 in subsequent examples. In addition, tumour tissue from a mastectomy sample stained strongly for GLUT8, but normal breast tissue from the same sample was negative, as 20 illustrated in Figure 10.

Example 9 Peptide Competition

The specificity of R1396 antiserum for immunocytochemical detection of GLUT8 was further tested in 25 MCF7 cells. Cells were grown, serum starved and fixed as described in Example 17 below. Insulin treatments were for 20 min at 1µM. Peroxidase staining was as described in Example 14 except for the omission of H₂O₂ in the detection. Competitive and non-competitive peptides 30 (60µg/ml in PBS) were incubated on cells for 1h at room temperature prior to addition of antisera (R1396 or pre-immune bleed) at dilutions of 1/300 or 1/100 containing competitive or non-competitive peptides (final concentration 60ug/ml) over-night at 4°C. Competitive 35 peptide was that used to immunise and non-competitive peptide EELVPKQPQKRPQELLEC.

These experiments confirm the specificity of R1396 antiserum. GLUT8 staining was observed in the perinuclear region of MCF7 cells. Staining pattern was similar but weaker in serial dilutions. No difference in staining pattern was observed following short-term insulin treatment. Staining was competed out by competitive but not non-competitive peptide as shown in Figure 11.

Example 10 Western Blot Analysis of the Antibody Raised
to the C-terminal Region of GLUT8

A polyclonal antibody specific to the C-terminus of the GLUT8 sequence was produced and tested in immunohistochemical and immunocytochemical experiments as described above. The antibody, and subsequently the GLUT8 protein, was further characterised by Western blotting experiments and by peptide affinity purification of the antisera.

(a) Affinity purification

20 Peptide affinity columns comprising 2mg of GLUT8
C-terminal peptide per 1ml column were prepared with
SulfoLink Coupling Gel according to the manufacturer's
instructions (Pierce Biochemicals, Rockford, Illinois).
Purified antibody was eluted with 0.2M glycine, pH2.0 and
25 dialysed against PBS.

(b) Western Blot Analysis

Crude protein extracts were prepared from MCF7 cultured malignant breast epithelial cells using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Protein was assayed by the BIORAD protein detection method and protein (80µg), analysed by SDS PAGE on a 12% resolving gel. Proteins were immunoblotted using GLUT8 antisera (1:500) or affinity-purified antibody (50µgml), and immunolabelled proteins visualised using chemiluminescence detection (Boehringer Mannheim).

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Both GLUT8 antisera and affinity-purified antibody immunolabelled a specific protein species with an approximate mobility of 50kDa, and also labelled a slightly faster-migrating species. These results are shown in Figure 12. GLUT8 protein has been demonstrated in MCF7 and T47-D cultured malignant breast epithelial cells. The mammalian glucose transporter proteins range from 492 to 524 amino acids (Bell et al., 1990).

10 Example 11 Deglycosylation Experiments and Membrane Preparations

To test the hypothesis that both protein species detected by the GLUT8 antiserum could be different forms of the same protein, protein extracts (80µg) were treated with endoglycosidase H, 2 mU (Boehringer Mannheim) for 16h, pH5.2, 37°C. Western blots of endoglycosidase-treated samples were immunoblotted with GLUT8 antiserum. Only the faster migrating of the two protein species was present in treated samples (Figure 13), indicating that the GLUT8 protein is glycosylated and that the two proteins detected on Western blots may be different glycosylated forms of the GLUT8 protein. All the members of the facilitative glucose transporter family possess potential sites for N-linked glycosylation. Mutation of Asn-45 of GLUT1 increases the Km for glucose by 2 fold, indicating that glycosylation of the transporter proteins may be necessary for efficient glucose transport (Bell et al. 1993). It is not known at this stage why two potentially altered glycosylated forms of the GLUT8 protein can be detected in MCF7 cells. However, it is noted that during glucose starvation of 3T3-L1 adipocytes, an aglyco form of GLUT1 accumulates (Muekler 1993). The molecular size of GLUT1, which is the predominant transporter in L6 myoblast cells, is greater than that in differentiated myocytes, where GLUT4 is the functional transporter, and this discrepancy is thought to be a result of glycosylation of the GLUT1 protein (Mitsumoto and Klip, 1992).

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The glucose transporter proteins are membrane-associated, and can be extracted in membrane fractionation experiments (Bell et al. 1990, Walker et al. 1990). In order to determine whether GLUT8 was present in the membrane fraction of the protein extracts, crude protein extracts from MCF7 cells were precipitated with 1M KCl for 30 min on ice. Following centrifugation at 14,000 rpm for 30 min, the crude total membrane fraction was solubilized in detergent (10mM Tris pH8.0, 5mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X100).

Membrane fractions were subjected to Western analysis. The GLUT8 polyclonal antibody immunolabels two protein species at approximately 50kDa in membrane fractions of these cells as shown in Figure 13.

15

Example 12 Cross-Reactivity of GLUT4 Polyclonal Antibody

As discussed above, GLUT8 was unexpectedly identified as a GLUT4-like transporter in malignant breast epithelial cells. In order to clarify whether both GLUT4 and GLUT8 were expressed in these cells, a monoclonal antibody directed against GLUT4 (mAb 1F8; James et al. 1988) was used in Western blot analysis. 1F8 does not detect an immunoreactive protein of 50kDa in MCF7 protein extracts. Rat gastrocnemius muscle protein extracts were used as a positive control in these experiments, as 1F8 cross-reacts with both human and rat GLUT4 (Fukumoto et al. 1989).

The results of these experiments, illustrated in Figure 14, suggest that the C-terminal directed GLUT4 polyclonal antibody (R820) cross-reacts with both GLUT4 and GLUT8, but that the GLUT8 polyclonal antibody (R1396) described herein is specific for the GLUT8 protein.

35 Example 13 Tissue Distribution of GLUT8 Expression

Because only low levels of GLUT8 messenger RNA was detected in all tissues tested, the tissue distribution

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of expression of GLUT8 was further characterised by Western blot analysis.

Crude protein extracts were prepared from human tissues using the Trizol extraction method described above. Membrane fractions were prepared as described in Example 11, and immunoblotted with GLUT8 antiserum. We found that GLUT8 protein was present in human adipose tissue and skeletal muscle (Figure 16a), but not in brain, liver, or kidney. GLUT8 immunoreactive protein was also detected in human small intestine protein extracts. It is not known at this stage if the GLUT8 protein in small intestine is present in the mucosa or in smooth muscle remaining after dissection. Our results from immunohistochemical analysis of breast tumour samples suggest that GLUT8 is present in smooth muscle cells surrounding blood vessels (Example 14).

It is of importance for future studies of GLUT8 to determine if the R1396 polyclonal antibody immunolabels GLUT8 protein from other species. Protein was extracted from rat adipose tissue and skeletal muscle (gastrocnemius) by Trizol reagent, and 40µg protein separated by SDS PAGE. Proteins were immunoblotted with GLUT8 antiserum (1:500). GLUT8 protein was detected in both the rat adipose tissue and skeletal muscle as shown in Figure 15.

To confirm the presence of GLUT8 protein in skeletal muscle, sections of paraffin-embedded human skeletal muscle (*brachioradialis*, female) were subjected to immunohistochemical staining with GLUT8 polyclonal antiserum as described in Example 14. Muscle fibre bundles demonstrated strong staining of GLUT8 protein, with no staining by non-immune serum control serum (Figure 16b).

Example 14 Use of GLUT8 Antibody for Studying
Expression of GLUT8 in Breast Tumours

The histopathology of the breast tumour sample shown in Figure 10 has been confirmed as ductal cell carcinoma *in situ* (DCIS). No GLUT4 protein could be detected in this tumour by immunohistochemistry using 1F8 GLUT4 monoclonal antibody. This result is significant in

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that it indicates that the GLUT8 antibody R1396 is suitable for studying expression of GLUT8 in breast tumour samples. DCIS is a very early stage of breast tumour progression. Detection of GLUT8 in this tumour type suggests that GLUT8
5 may be involved in supply of energy to cells at a very early stage of tumour progression, and ultimately therefore may provide a means of early detection.

In order to investigate this observation further, RT-PCR using specific primer pair 2 was used to compare
10 expression of GLUT8 in 10 breast cancer patients. Sample collection and PCR conditions are as described in Example 7; however the Expand High Fidelity PCR System (Boehringer) was used for amplification of GLUT8. RNA was extracted by Trizol (Life Technologies). In addition, paraffin sections
15 of the tumour samples were stained for immunohistochemical detection of GLUT8 using the standard peroxidase-labelled streptavidin-biotin detection method (Boenisch, 1989). R1396 GLUT8 polyclonal antiserum was incubated on sections overnight, 4°C at a dilution of 1/300. The peroxidase
20 activity was detected with 3'-3'-diaminobenzidine tetrahydrochloride (Sigma) and H₂O₂ (0.15%). Counterstain was haematoxylin.

Patient demographics are described in Table 1a and results of RT-PCR and immunohistochemistry summarized
25 in Table 1b. Comparison of fold increase of GLUT8 expression in tumour tissue over normal breast tissue from the same patient by RT-PCR showed that in 5 of the 10 patients studied, GLUT8 levels were increased in tumour samples. Increases ranged from 3 to 20 fold.
30 Immunohistochemical detection allowed comparison of GLUT8 levels in tumour cells and normal ducts at tumour margins. In all but one of the ten patients studied, immunohistochemical and RT-PCR results showed corresponding increases of GLUT8 levels in tumour cells compared to
35 normal ducts. The DCIS component of 6 tumours stained strongly for GLUT8. Smooth muscle cells lining blood vessels were noted to stain positive for GLUT8.

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Patient	Age	Size of tumour	Nodes +ve	Histology	Grade	E.R.	P.R.
1	25	12mm	0	APO	3	-	-
2	42	13mm	0	IDC	2	+	+
3	39	29mm	2	IDC	3	+	+
4	56	27mm	0	IDC	2	+	+
5	85	47mm	4	IDC	3	-	+
6	52	10mm	0	LOB	2	+	+
7	62	16mm	1	IDC	1	+	+
8	77	22mm	0	IDC	2	+	+
9	70	38mm	15	IDC	3	+	+
10	85	26mm	1	IDC	3	-	-

Patients demographics

ABBREVIATIONS : E.R : oestrogen receptor, P.R. : progesterone receptor

APO : apocrine carcinoma, IDC : intraductal carcinoma, LOB : lobular carcinoma

ABBREVIATIONS : DCIS : ductal cell carcinoma *in situ*,
1+ faint, 2+ medium and 3+ strong staining

Patient	PCR fold increase	Invasive Strength	Invasive Area	DCIS Strength	DCIS Area	Normal	Comments
1	1	2+	50%	2+	60%	10% ducts 10% lobules	(myoepithelial cells staining)
2	6	1+	70%	2+	70%	55% ducts 10% lobules	
3	1.4	2+	10%	1+	5-10%	-ve	(cysts of the fibrocystic disease are staining)
4	19.7	2+	60%	2+	60%	20% ducts 20% lobules	
5	18.3	3+	>90%	3%	90%	40% ducts 20% lobules	
6	1.3	1+	40%	not seen		5-10% ducts 50% lobules	(cysts of fibrocystic disease are staining)
7	3	1+	10-15%	3+	>90%	5-10% ducts 50% lobules	
8	1	1+	<10%	not seen		20% ducts	
9	3.6	3+	75%	3+	75%	10% ducts 50% lobules	
10	1		0	0	0	5-10% ducts 5-10% lobules	

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Example 15 In vitro Transcription/Translation

The following experiments were performed in order to:

- (a) confirm the size of the GLUT8 protein produced from the cloned cDNA; and
- (b) confirm that the GLUT8 protein can be glycosylated.

In vitro transcription/translation experiments were performed using a rabbit reticulocyte lysate system (Promega) according to the manufacturer's instructions. *In vitro* transcription/translation of the GLUT8 cDNA clone yielded a polypeptide of similar mobility (approximately 40kD) to that produced from rat GLUT4 cDNA. In addition, translation of GLUT4 and GLUT8 mRNA in the reticulocyte lysate system, in the presence of pancreatic microsomal membranes (Promega), reduced the mobility of both protein products, with the larger translation products being present in the pelleted microsome membrane fraction. This is illustrated in Figure 17. These results suggest that co-translational glycosylation of the translation products had occurred under these conditions, and therefore correlate with our results from the deglycosylation experiments described in Example 11.

Example 16 Effect of Insulin on Cellular Localisation of GLUT8 in MCF7 Cells

To investigate the effect of insulin on cellular localisation of GLUT8 in MCF7 cells, cells were incubated in 10 nM insulin for 15 min. and then subjected to immunocytochemical staining as described in Example 8 and 9. In this case the serum dilution was 1:300. The results are shown in Figure 18. Non-immune serum again showed no staining, and in the absence of insulin strong staining was observed. After incubation with insulin the staining was diffuse and less intense. It is unclear at this stage whether this indicates movement of GLUT8 into other cell compartments or to the plasma membrane.

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Example 17 Immunofluorescence Studies of GLUT8 Cellular
Localisation

As GLUT8 was originally identified on the basis of its homology to GLUT4, and has now been shown to be
5 expressed in insulin-sensitive tissues (skeletal muscle and adipose tissue), we further investigated the potential for translocation of GLUT8 protein in response to insulin treatment. These experiments were performed with cultured malignant breast epithelial cells, but may also be
10 performed with insulin sensitive adipose tissue and muscle cells. Previous results using peroxidase-antiperoxidase staining techniques demonstrated a different staining pattern in MCF7 cells after exposure to insulin. Therefore the more sensitive technique of immunofluorescence was used
15 to determine whether GLUT8 protein is translocated in response to insulin treatment.

Cells were grown in RPMI medium supplemented with 10% Fetal Calf Serum (FCS) and 50nM insulin. Following fixation with paraformaldehyde (4% in RPMI medium), cells
20 were quenched in glycine (100mM), permeabilized with 0.1% Triton X-100 and blocked in horse serum (2%). Cells were incubated overnight at 4°C with R1396 GLUT8 antiserum (1/300 in 0.2% horse serum), washed with PBS and incubated for 1h with Texas Red-X goat anti-rabbit IgG, 5µg/ml
25 (Molecular Probes, USA). For short-term insulin treatment, cells were incubated in RPMI supplemented with 2% FCS for 16h prior to treatment and then in RPMI supplemented with 0.2% BSA for 1h immediately prior to addition of insulin. For long-term insulin treatment, cells were maintained in
30 RPMI supplemented with 10% FCS and 50nM insulin. Where the combined effects of serum and insulin were compared to either serum or insulin alone, cells were grown in RPMI with 10% FCS and 50nM insulin, with media then changed to RPMI with 0.2% BSA, RPMI with 0.2% BSA and 50nM insulin or
35 RPMI with 10% FCS respectively, and cells incubated for a further 16h prior to fixation.

The cellular localisation of GLUT8 protein was

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examined in MCF7 cells by immunofluorescence confocal microscopy. When MCF7 cells were incubated under basal, serum and insulin-starved conditions, staining for GLUT8 was in a tight peri-nuclear pattern with no plasma membrane staining. Growing cells continuously in the presence of insulin (50nM) and 10% FCS, resulted in a different distribution of GLUT8 protein, with staining throughout the cell and at the plasma membrane. No staining was observed in cells treated with pre-immune serum. These results, shown in Figure 19, may be indicative of intracellular sequestration of GLUT8 protein in the absence of hormonal stimuli. When MCF7 cells were incubated in either RPMI and 10% FCS or in medium supplemented with insulin alone, the staining pattern observed was similar to that observed under basal, serum and insulin-starved conditions, suggesting that the presence of insulin and an as yet unidentified serum component is required for the redistribution of GLUT8 in these cells.

In insulin-sensitive muscle cells and adipose tissue in the absence of insulin, GLUT4 is sequestered to intracellular compartments. On exposure of these cells to insulin, GLUT4 is rapidly translocated to the plasma membrane, resulting in a rapid and large increase in glucose transport (reviewed by Bell et al. 1993). Insulin has been shown to stimulate cell growth of MCF7 cells via the insulin receptor, which is present at elevated levels in these cells compared to normal breast epithelial cells (Milazzo et al 1992). In our experiments with MCF7 cells we have been unable to demonstrate any acute increase in GLUT8 labelling at the cell surface after exposure to insulin for 15 to 60 minutes at concentrations ranging from 10 nM to 1 μ M. Glucose uptake over this time-frame is elevated 3-fold with insulin treatment, but this relatively small increase could be explained by either increased activity or increased plasma membrane levels of GLUT1, as this GLUT is expressed at high levels in MCF7 cells. However, there is clearly an altered subcellular location

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of GLUT8 protein when cells are exposed to insulin over longer incubation periods of up to 5 days. Therefore it is possible that this redistribution represents a form of protein trafficking.

5 Altered cellular localisation of GLUT8 in response to insulin and/or other factors may provide a control mechanism for growth of breast cancer cells. In addition we have demonstrated that GLUT8 is present in muscle cells and adipose tissue. These tissues, which
10 express the GLUT4 isoform, are described as classically insulin-responsive. Specific sequences have been identified in the N- and C-terminal regions of GLUT4 which are thought to direct cellular localisation and trafficking in response to insulin (Piper et al. 1993). It is thought
15 that reduced insulin-responsiveness in NIDDM may be a result of defective insulin-stimulated translocation of the GLUT4 protein. Much progress has been made in the understanding of the intricate molecular mechanisms which control this process. However, defects in GLUT4
20 translocation may not be sufficient to cause hyperglycaemia. Recent data arising from the study of GLUT4 knock-out mice suggests that a novel insulin responsive glucose transport system may operate in soleus muscle in the absence of GLUT4 expression and under
25 conditions of hyperinsulinaemia (Stenbit et al 1996). We have shown that the novel glucose transporter-like protein GLUT8 of this invention is expressed in human skeletal muscle cells and adipose tissue, and therefore could play a role as a second or compensatory insulin responsive
30 transport system. Expression or activity of a second or compensatory insulin stimulated glucose transport system could be altered in insulin resistant NIDDM.

Example 18 Northern Blot Analysis of MCF7 Cells

35 Northern blot analysis of RNA extracted from MCF7 cells detected specific transcripts of approximately 4.4 and 2.5 kb that hybridise to the GLUT8 cDNA, as shown in

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Figure 20. The two transcripts that hybridised to the GLUT8 cDNA on Northern blots of MCF7 mRNA may reflect differing lengths of untranslated regions, as has been reported for the human GLUT5 isoform (Kayano et al. 1990).

5 The level of GLUT8 mRNA present in MCF7 cells is low, as transcripts could be detected in polyA but not in total RNA preparations. Whether this result represents a truly low level of mRNA expression in these cells, or whether the message is unstable, or has a high turnover mechanism of

10 regulation, requires further experimentation. However GLUT8 protein is readily detectable in MCF7 cells.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding,

15 various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

Reference cited herein are listed on the

20 following pages, and are incorporated herein by this reference.

REFERENCES

- Bell et al. 1990 Diabetes Care:13, 198-208.
- Bell et al. 1993 J. Biol. Chem.:268, 19161-19164
- Boenisch, T. 1989 Handbook: Immunochemical
- 5 Staining Methods, DAKO Corporation
- Chomcynski and Sacchi 1987 Anal. Biochem.: 162, 156-159
- Fukumoto et al. 1989 J. Biol. Chem.:264, 7776-7779
- 10 Hatt et al. 1992 Strategies: 5, 2-3.
- Kahn 1994 Diabetes: 43, 1066-1084.
- James et al. 1988 Nature: 333, 183-185
- James et al 1989 Nature: 338: 88-87.
- Kayano et al. 1990 J. Biol. Chem.: 265, 13276-13282
- 15 Keydar et al 1979 Eur. J. Cancer: 15, 659-670.
- Labora, J. 1991 Nucl Acids Res.: 19, 3998
- Mitsumoto and Klip 1992 J. Biol. Chem.:267, 4957-4963
- 20 Millazo G 1992 Cancer Res.: 52, 3924-3930.
- Muekler 1993 J. Diab. Compl.:7, 130-141
- Piper et al. 1993 J. Cell Biol.: 121, 1221-1232.
- Sanbrook et al 1989 Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory
- 25 Press
- Soule et al 1973 J. Natl. Cancer Inst.: 51, 1409-1416.
- Stenbit et al 1996 J. Clin. Invest.: 98, 629-634
- Verhey and Birnbaum 1994 J Biol Chem., 269, 2353-2356
- 30 Walker et al. 1990 J. Biol.: 265, 1516-1523

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A nucleic acid molecule encoding a facilitative glucose transporter protein (GLUT8) or functional fragment thereof.
2. A nucleic acid molecule according to claim 1, wherein the nucleic acid molecule is selected from the group consisting of genomic DNA, cDNA, and RNA.
3. A nucleic acid according to claim 2, wherein the nucleic acid is cDNA.
4. A nucleic acid according to claim 3, wherein the nucleic acid has the sequence as set out in SEQ ID NO:4.
5. A nucleic acid molecule or fragment thereof which hybridizes under stringent conditions to the sequence set out in SEQ ID NO:4.
6. A facilitative glucose transporter protein (GLUT8) or biologically-active fragment thereof.
7. A transporter protein according to claim 6, wherein the protein has greater than 70% sequence homology with the amino acid sequence set out in SEQ ID NO:5.
8. A transporter protein according to claim 7, wherein the protein has greater than 80% sequence homology with the amino acid sequence set out in SEQ ID NO:5.
9. A transporter protein according to claim 8, wherein the protein has greater than 95% sequence homology with the amino acid sequence set out in SEQ ID NO:5.
10. A method for diagnosing a malignant condition, comprising the step of detecting expression or activity of GLUT8 in a tissue or cell.
11. A method of monitoring of efficacy of treatment of a malignant condition, comprising the step of detecting activity or expression of GLUT8 in a tissue or cell.
12. A method according to claim 11, wherein the method of detection of GLUT8 is selected from the group consisting of immunocytochemistry, hybridisation analysis, PCR and RT-PCR.
13. A method of selecting a method of treatment of a

malignant condition, comprising the step of measuring the ability of a proposed therapeutic agent to inhibit activity or expression of GLUT8 in a tissue or cell.

14. A method according to claim 13, wherein the inhibition of expression and/or activity of GLUT8 is brought about by either non-utilisable glucose analogues targeted to the malignant tissue or anti-sense nucleic acid sequences directed against the GLUT8 nucleic acid sequence.

15. A method according to any one of claims 8 to 11, wherein the tissue or cells are selected from adipose tissue or skeletal muscle cells.

16. A method according to any one of claims 11 to 15, wherein the malignant condition is selected from the group consisting of breast cancer, prostate cancer, epithelial cell cancers such as skin cancers and colon cancers.

17. An antibody directed against GLUT8, or a functional fragment thereof.

18. An antibody according to claim 17, wherein the antibody is either polyclonal or monoclonal.

19. An antibody according to claim 18, wherein the antibody is a polyclonal antibody directed against the C-terminal region of GLUT4.

20. An antibody according to any one of claims 17 to 19, wherein the antibody is directed against one or more epitopes present in the sequence set out in SEQ ID NO:12.

21. A method of treating non-insulin dependent diabetes mellitus, comprising the step of upregulating expression of GLUT8 in a tissue or cell.

22. A method according to claim 21, wherein the tissue is skeletal muscle and/or adipose tissue.

23. A method of detecting a mutation in the GLUT8 gene or regulatory sequence of a patient comprising the step of analysing the gene or regulatory sequence for a nucleic acid change compared to that set out in SEQ ID NO: 3 or 4.

24. A method according to claim 23, wherein the patient is a non-insulin dependent diabetes mellitus

patient and the nucleic acid is DNA.

25. A method according to claim 24, wherein the analysis is by single-stranded conformational polymorphism (SSCP).

5 26. A method of screening putative agents for treatment of cancer, comprising the step of measuring the ability of the agents to inhibit the activity of GLUT8 *in vitro* or *in vivo*.

27. A method according to claim 26, wherein the
10 method of screening is positron emission tomography scanning using a hexose labelled with a fluorescent marker.

28. A method according to claim 27, wherein the hexose is either a glucose analogue or hexose specifically transported by GLUT8.

15 29. A method of screening putative agents for treatment of diabetes and/or insulin-resistance syndrome comprising the step of measuring the ability of the agents to upregulate or enhance the activity of GLUT8 *in vitro* or *in vivo*.

20 30. A method according to claim 29, wherein the diabetes is non-insulin dependent diabetes mellitus.

31. A method according to claim 29, wherein the insulin-resistance syndrome is selected from the group
25 consisting of central obesity, hypertension, dyslipidaemia and glucose intolerance.

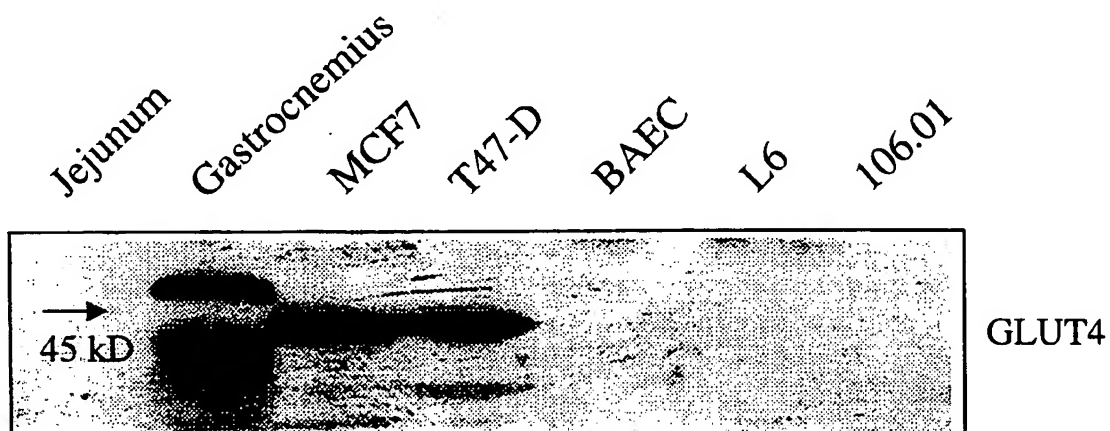


Figure 1a

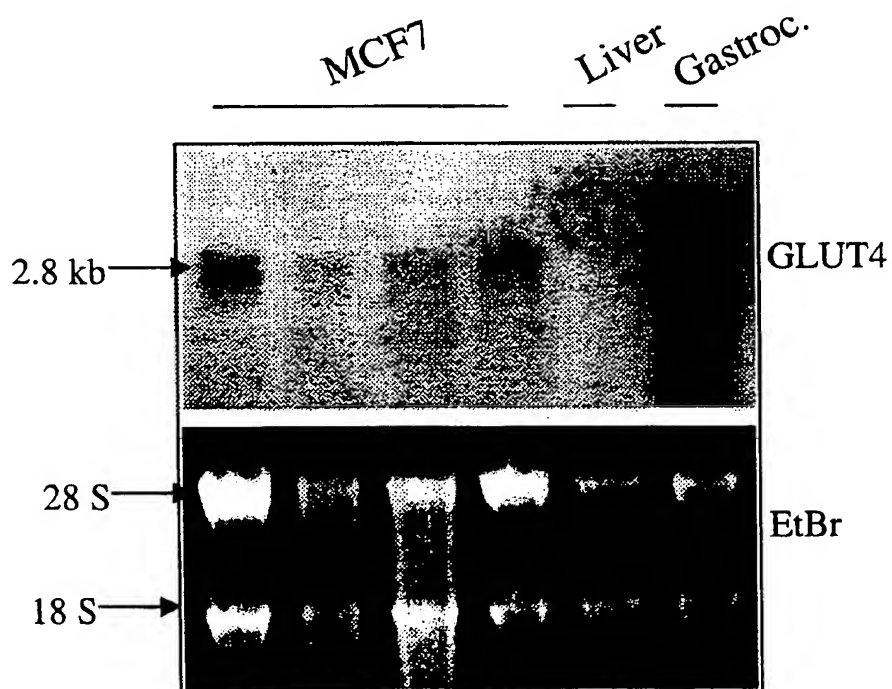


Figure 1b

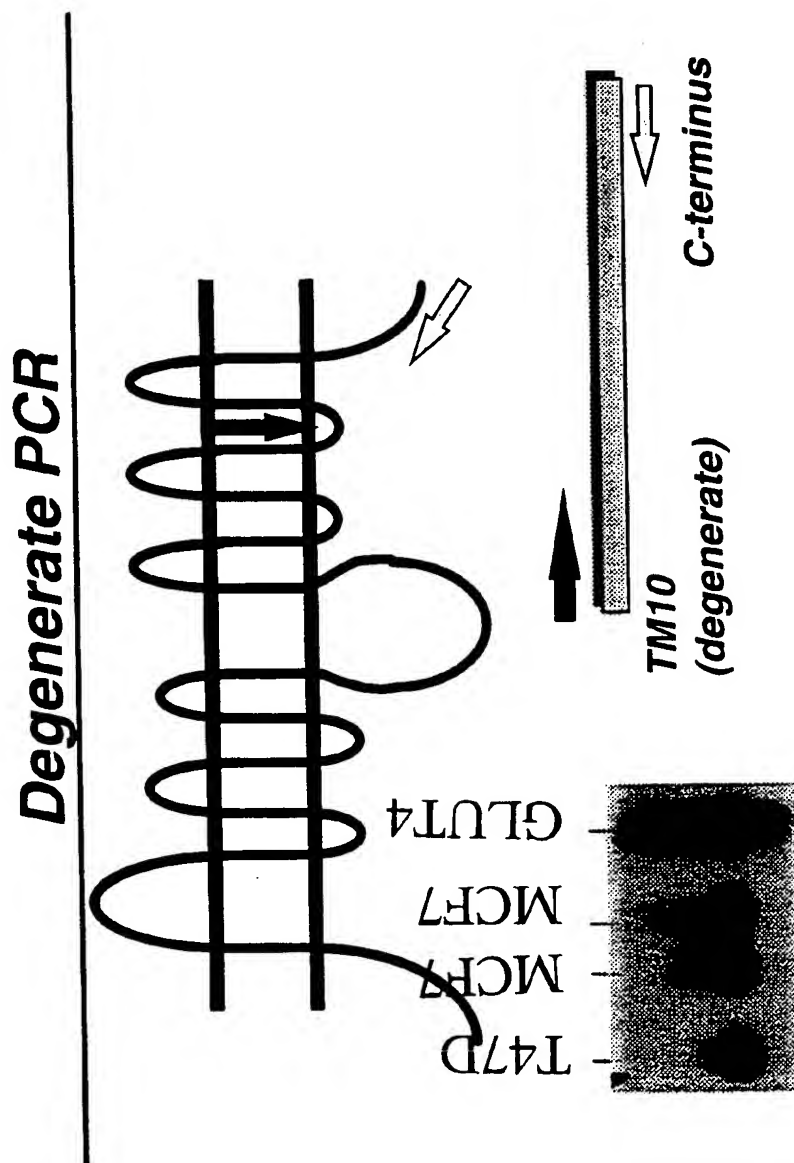


FIGURE 2

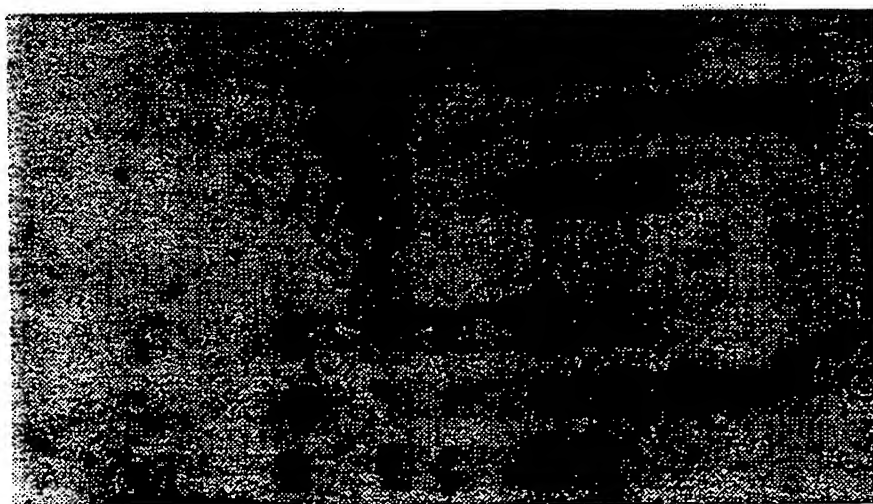
[illegible]

FIGURE 3

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Genomic Southern - probe GLUT8

Ec o R I P s t I



1. MCF7
2. Normal wbc
3. Normal wbc

FIGURE 4

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MCF7 Genomic DNA

- primer pair 1
- GLUT8 probe

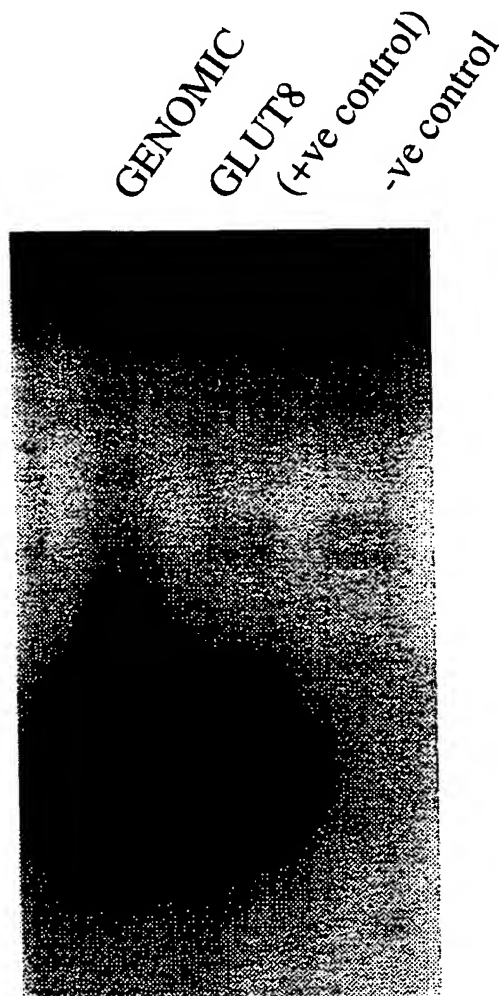


FIGURE 5

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G8 WKYMFGLVIPLGVLQAIAMYFLPPSPRFLVMKGQEGAASKVLGRLRALSDT
 G1 WPLLLSIIFIPALLQCIPLPFCPESPRFLLINRNEENRAKSVLKKLRGTAD
 G2 WHILLGLSGVRAILQSLLFFCPESPRYLYIKLDEEVKAKQSLKRLRGYDD
 G3 WPLLLGFTILPAILQSAALPFCPESPRFLLINRKEENAKQILQRLWGTQDV
 G4 WPLLLGLTVLPALLQLVLLPFCPESPRYLYIIQNLEGPARKSLKRLTGWAD
 G5 WPILLGLTGVPAAQLLLLPPFPSPRYLLIQKKDEAAAKKALQTLRGWDS
 M6 INTRACELLULAR LOOP

G8 . . TEELTVIKIKSSLKDEYQYSFWDLFRSKDNMRTRIMIGLTLVFFVQ
 G1 VTHDLQEMKEESRQMMREKKVTILELFRSPAYRQPILIAVVLQLSQQ
 G2 VTKDINEMRKEREASSEQKVSIIQLFTNSSYRQPILVALMLHVAQQ
 G3 VSQDIQEMKDESARMSQEKQVTVLELFRVSSYRQPIIISIVLQLSQQ
 G4 VSGVLAELKDEKRLERERPLSLLQLLGSRTHRQPLIIAVVLQLSQQ
 G5 VDREVAEIRQEDEAEKAAGFISVLKLFMRSLRWQLLSIIVLMGGQQ
 M7

G8 ITGQPNILFYASTVLKSVGFQSNEAASLASTGVG'VKVISTIPATLLVDREGRR
 G1 LSGINAVFYYSTSIFEKAGVQQP.. VYATIGSGIVNTAFTVVSFLVVERAGRR
 G2 FSGINGIFYYSTSIFQTAGISKP.. VYATIGVGAVNMVFTAVSVFLVEKAGRR
 G3 GINAVFYYSTGIFKDAGVQEP.. IYATIGAGVNTIFTVVSFLVERAGRR
 G4 LSGINAVFYYSTSIFETAGVGQP.. AYATIGAGVNTVFTLVSVLLVERAGRR
 G5 LSGVNAIYYYADQIYLSAGVPEEHVQ YVTAGTGAVNVVMTFCAV'VVELLGRR
 M8

FIGURE 6

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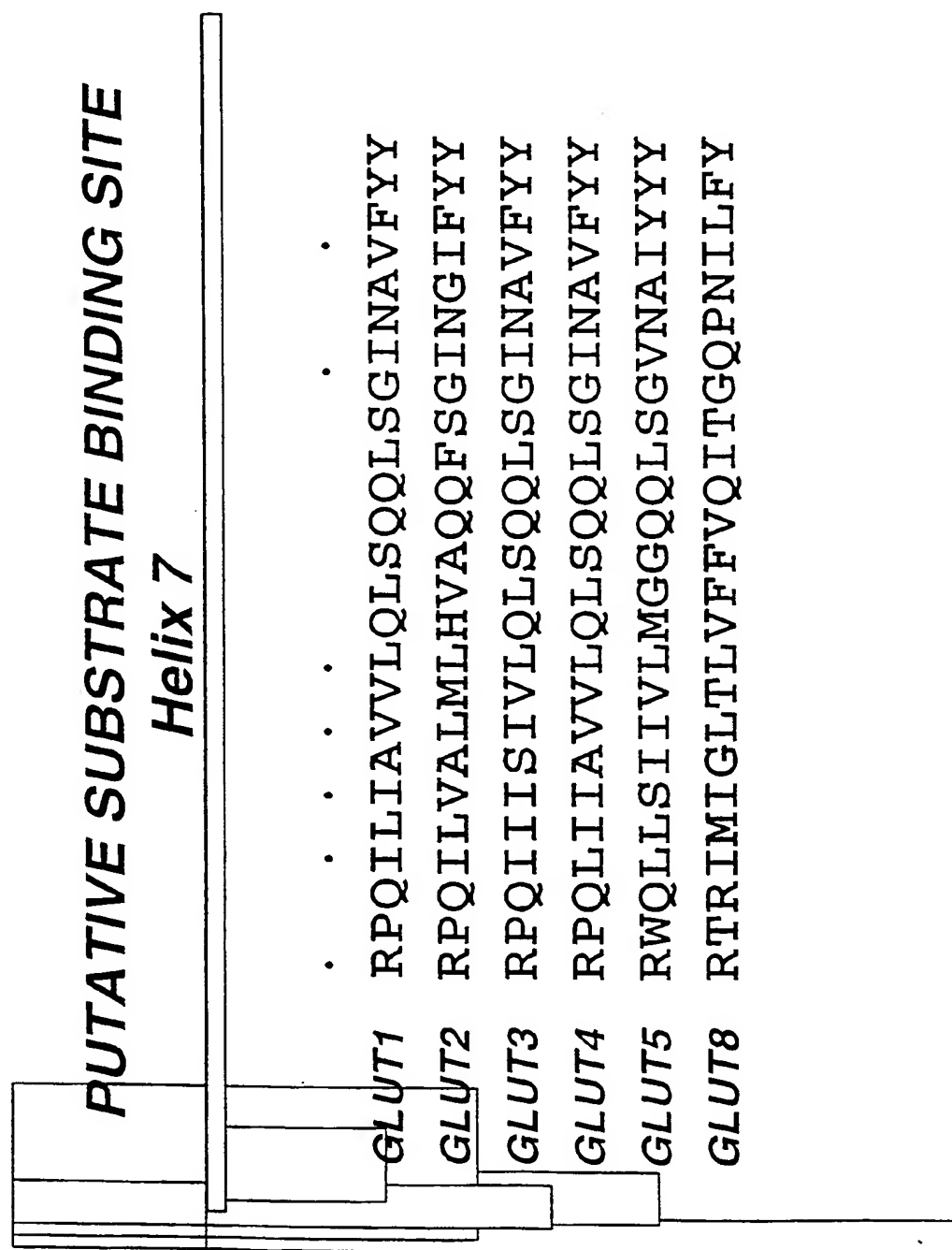


FIGURE 7A

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CYTOCHALASIN B BINDING SITE

G1	(F)EVGP	PIPW	FIVAE	LSQGP	RPAA	IAVAG	FSNW	TSNF	IVGMC	FQYV
G2	(F)EIGGP	PIPW	FMAEF	FSQGP	RPAA	LAIAA	FSNW	TCNF	IVALC	FQYI
G3	(F)EIGGP	PIPW	FIVAE	LSQGP	RPAA	MAVAG	CNSW	TSNFL	VGLLP	PSA
G4	(F)EIGGP	PIPW	FIVAE	LSQGP	RPAA	MAVAG	FSNW	TSNF	IIGMG	FQYV
G5	HALGPS	PIPAL	LITEI	FLQSS	RP	SAFM	VGGSV	HWLS	NFTV	GLIFPFI
G8	(F)SIGL	GPMP	WLVL	SEIF	PGGIR	GRAM	ALTSS	MNWG	INLLIS	LTLTLTV

-----M10----> <-----M11----->

FIGURE 7B

9/23

HOMOLOGY TO OTHER HEXOSE TRANSPORTERS

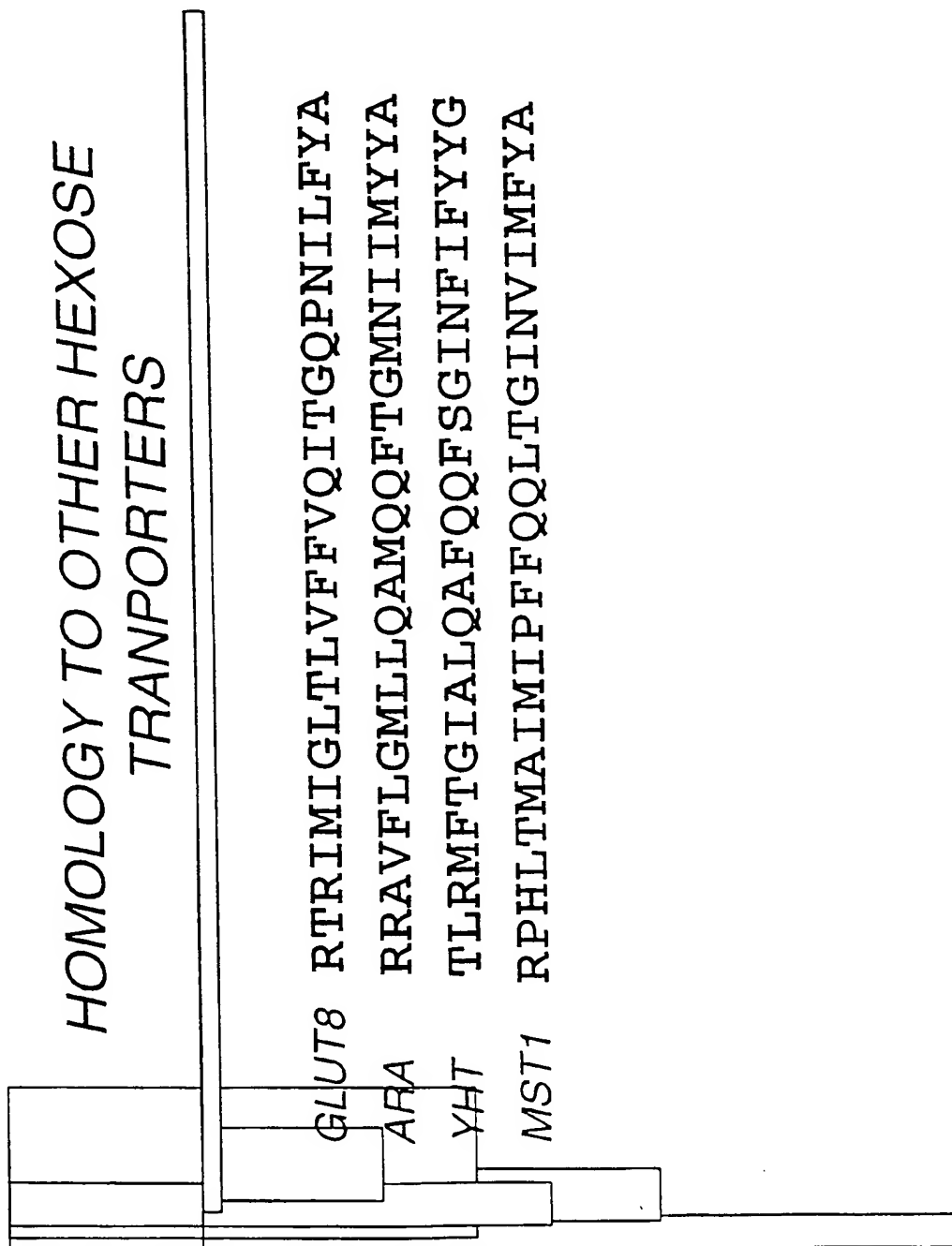


FIGURE 7C

10/23

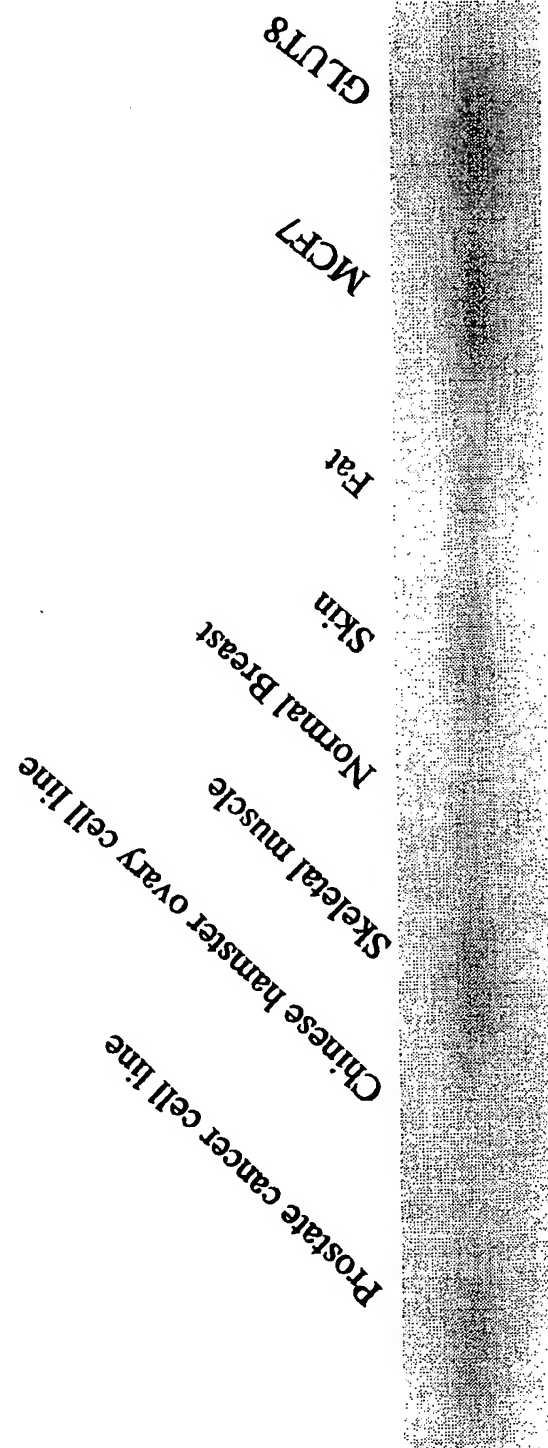
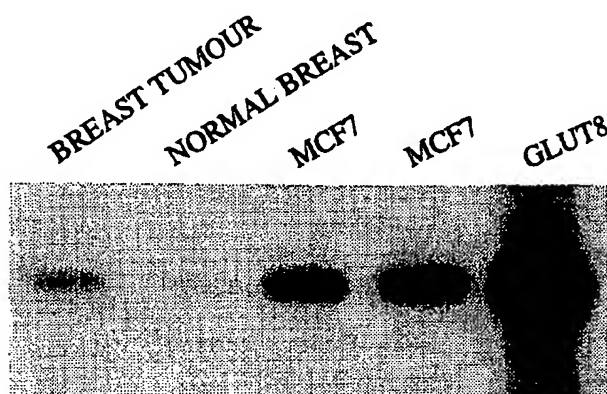
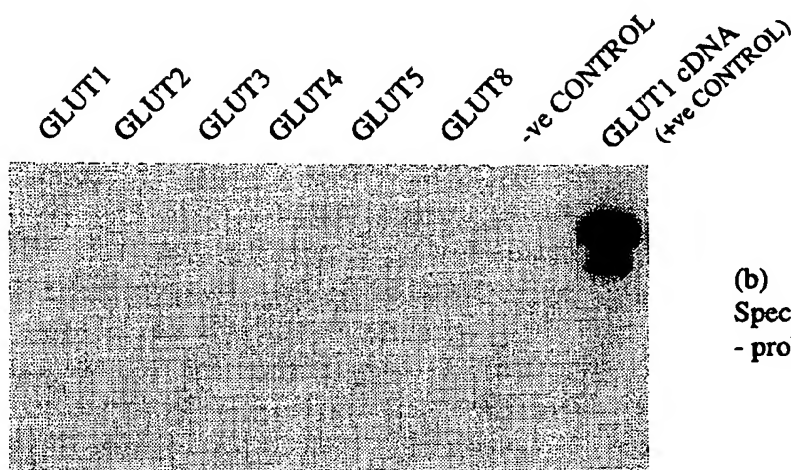


FIGURE 8

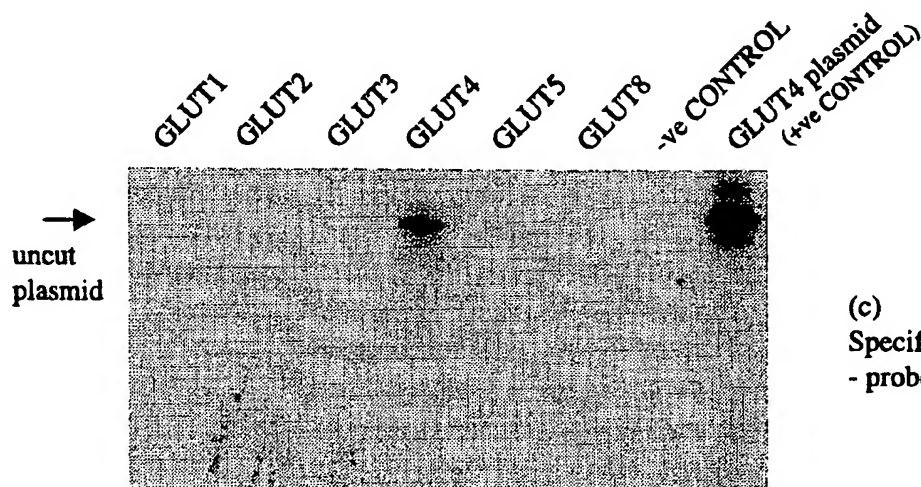
11/23



(a)
Specific primers for GLUT8
- probed with GLUT8



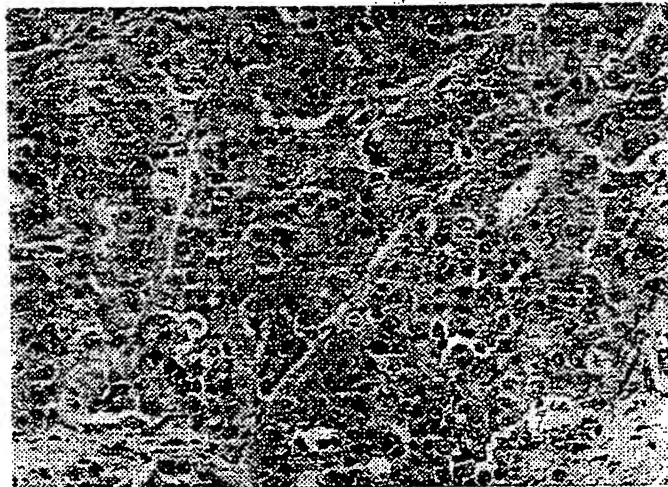
(b)
Specific primers for GLUT8
- probed with GLUT1



(c)
Specific primers for GLUT8
- probed with GLUT4

FIGURE 9

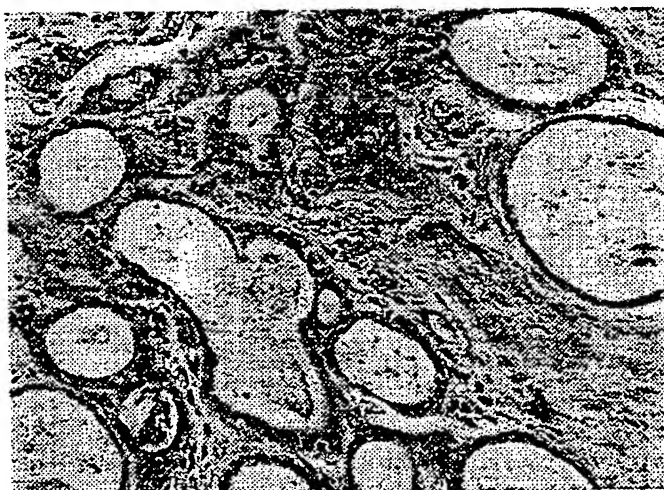
12/23



(a)
Non-immune
Breast tumour



(b)
Immune
Breast tumour



(c)
Immune
Normal Breast Tissue

Figure 10 : DCIS

13/23

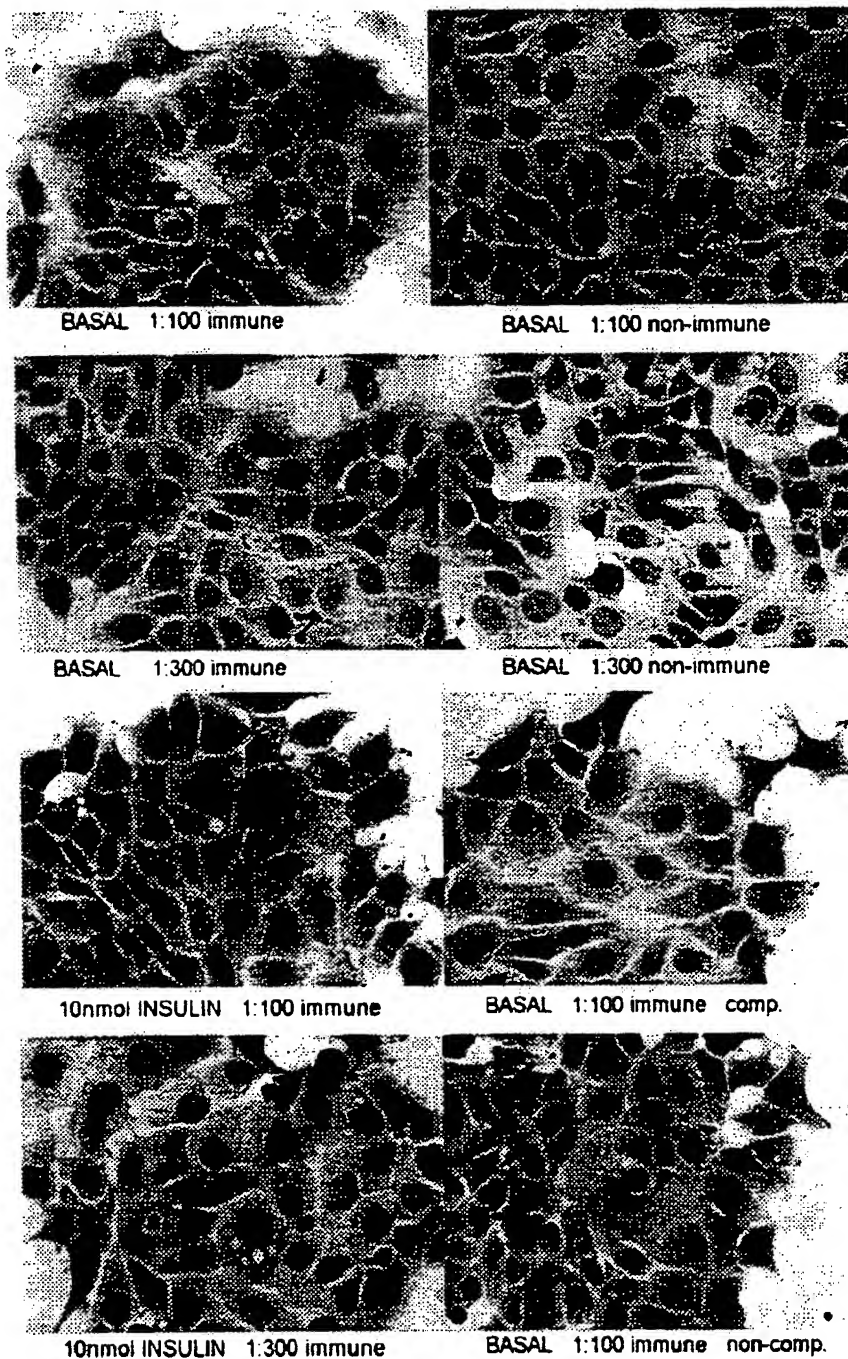


FIGURE 11

14/23

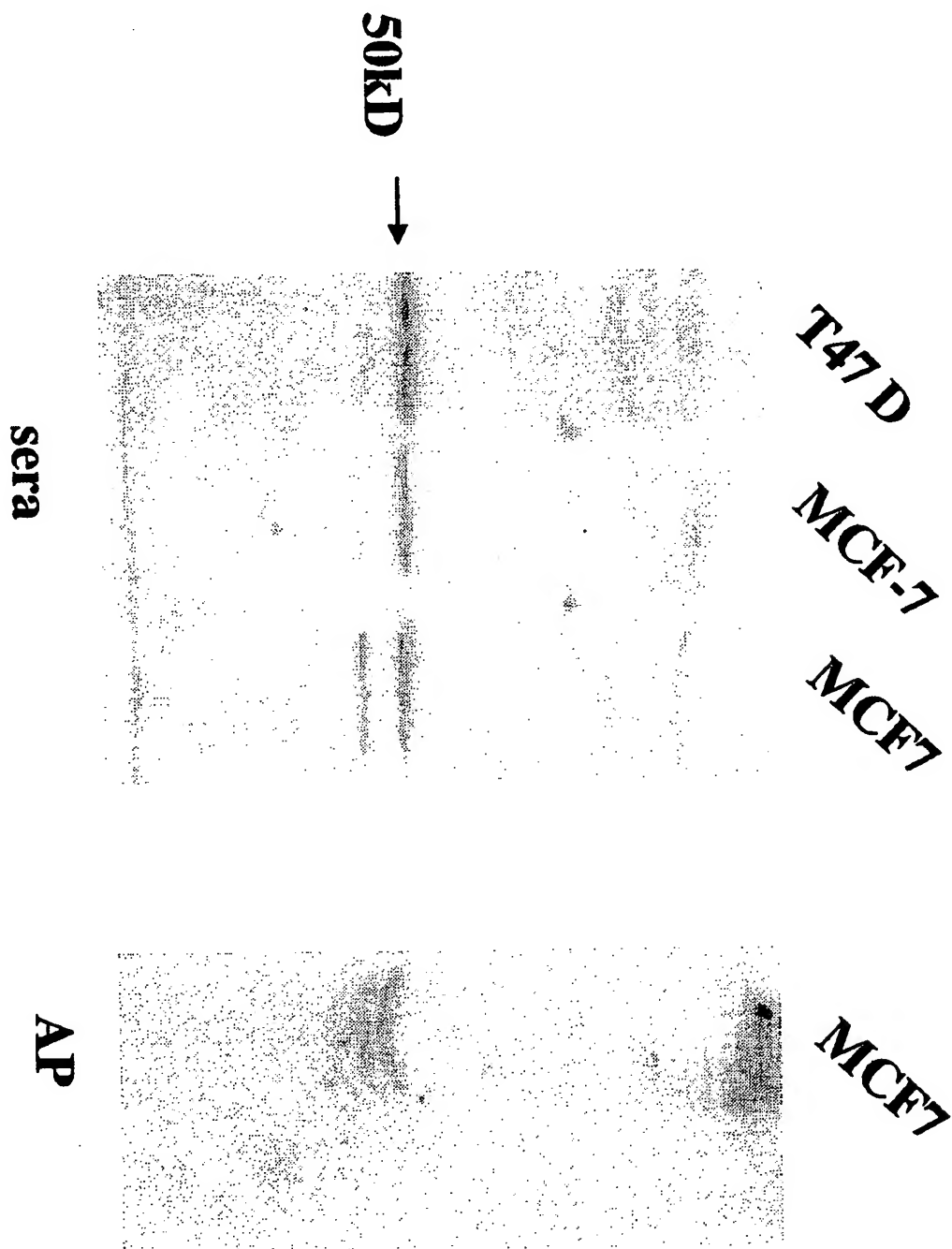


FIGURE 12

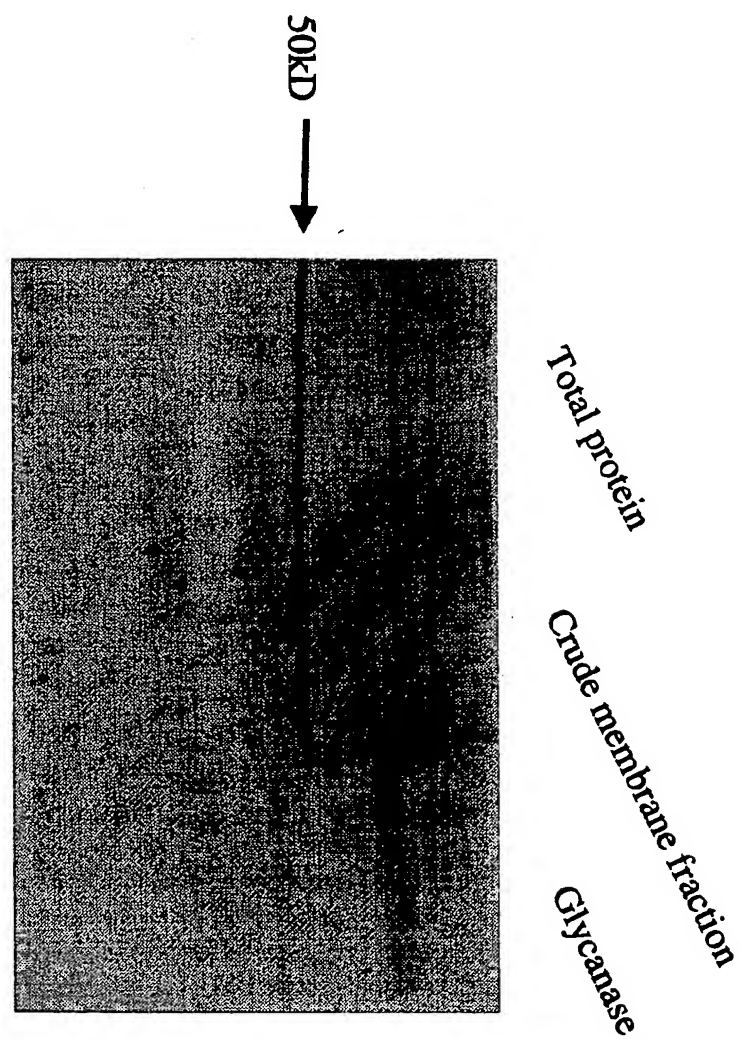
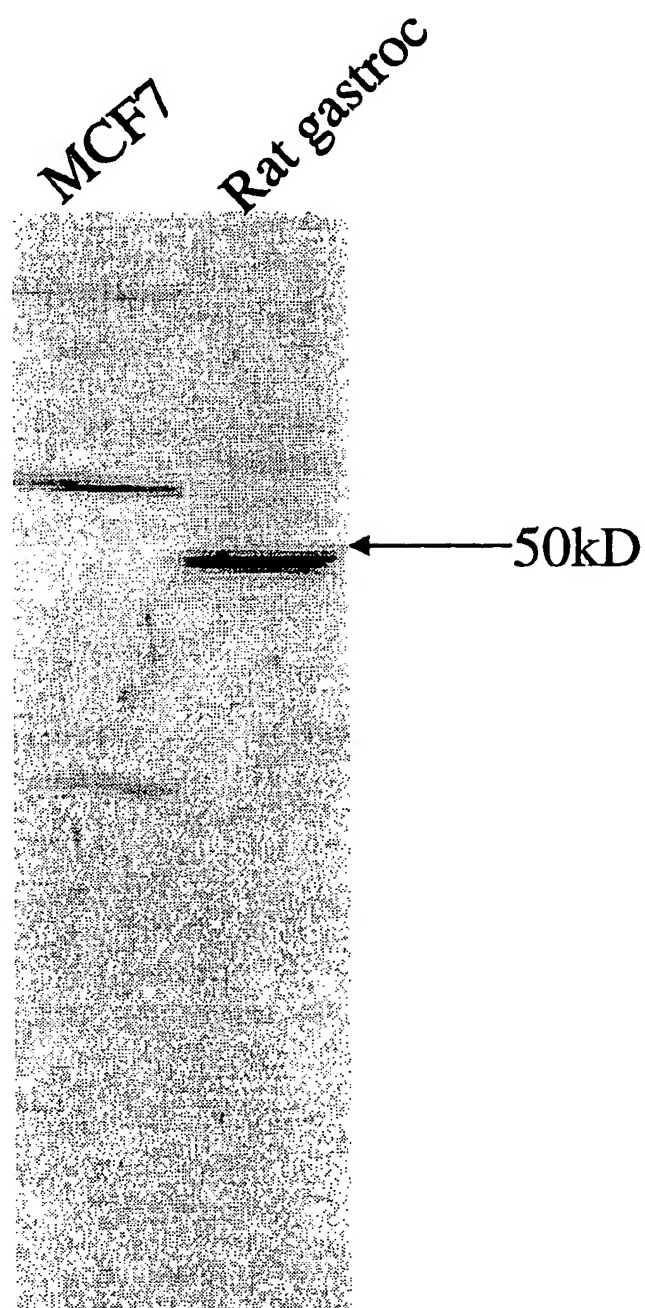


FIGURE 13

16/23



Western 1F8 GLUT4
monoclonal

FIGURE 14

17/23

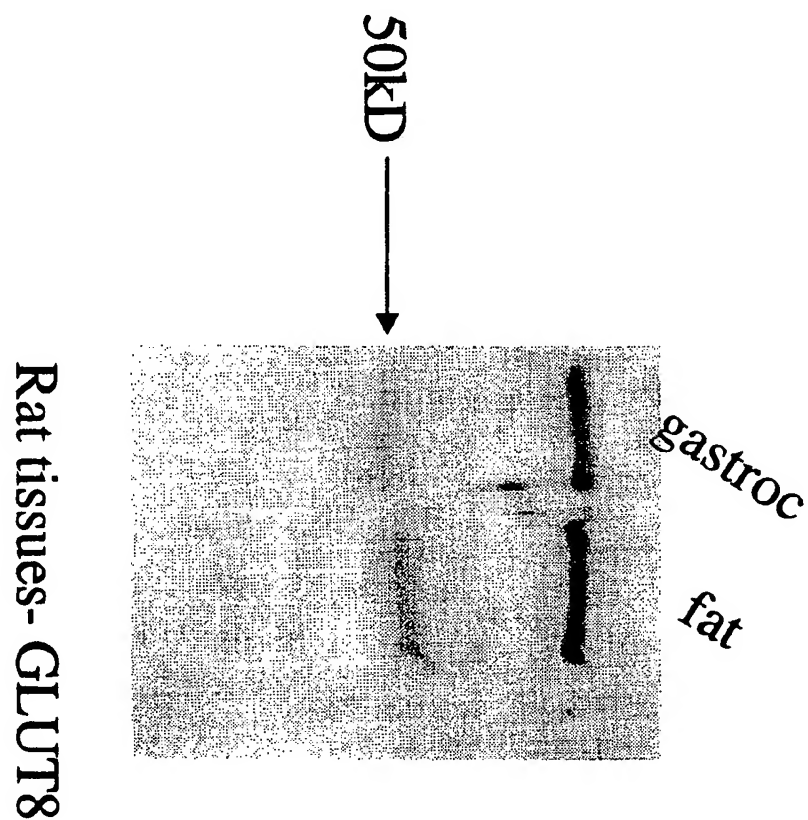


FIGURE 15

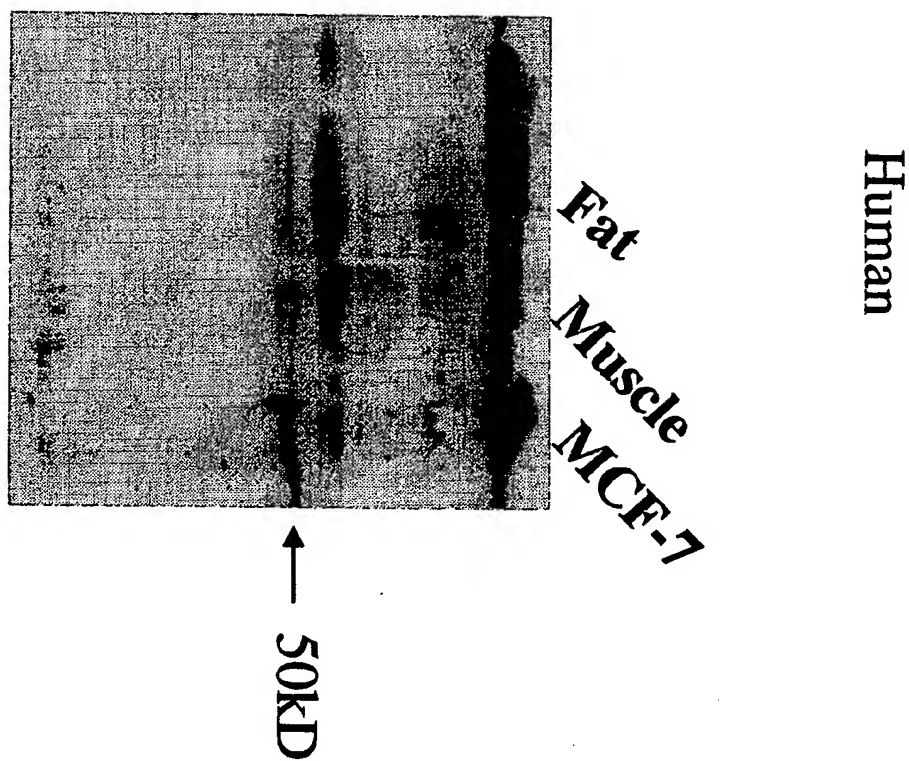
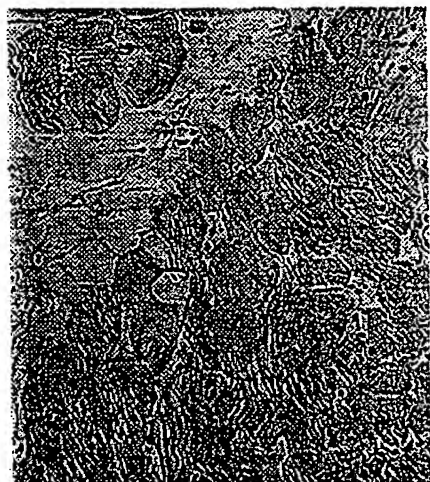


FIGURE 16A

19/23

HUMAN SKELETAL MUSCLE



Non-immune



10X



20X

GLUT8

FIGURE 16B

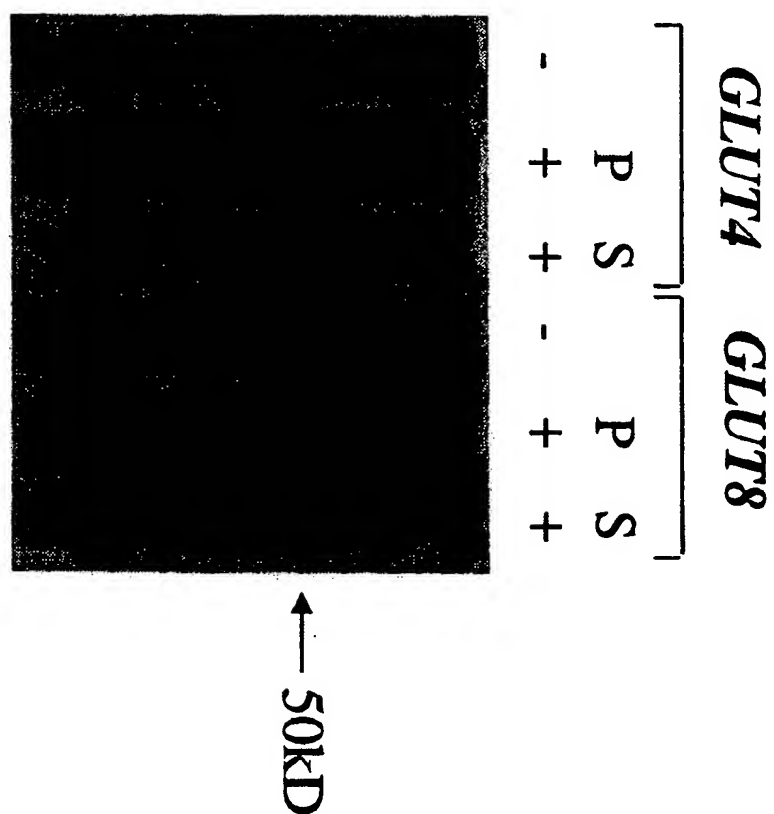
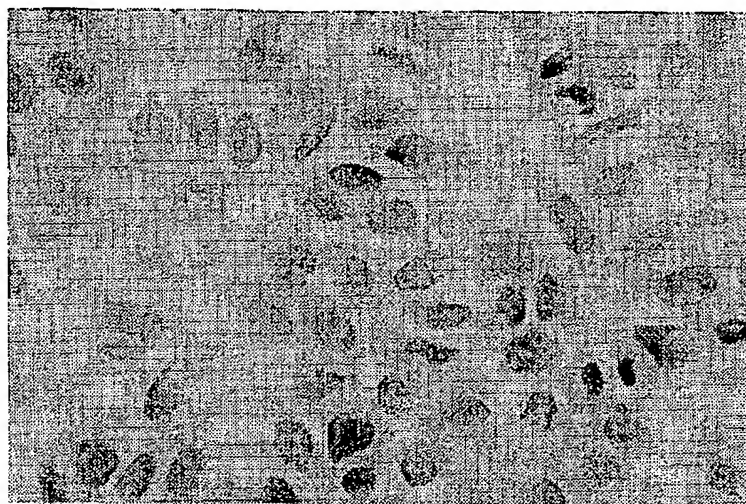


FIGURE 17



(a)
non-immune



(b)
no insulin



(c)
+ 10nM insulin
15min

Figure 18 : MCF-7

22/23

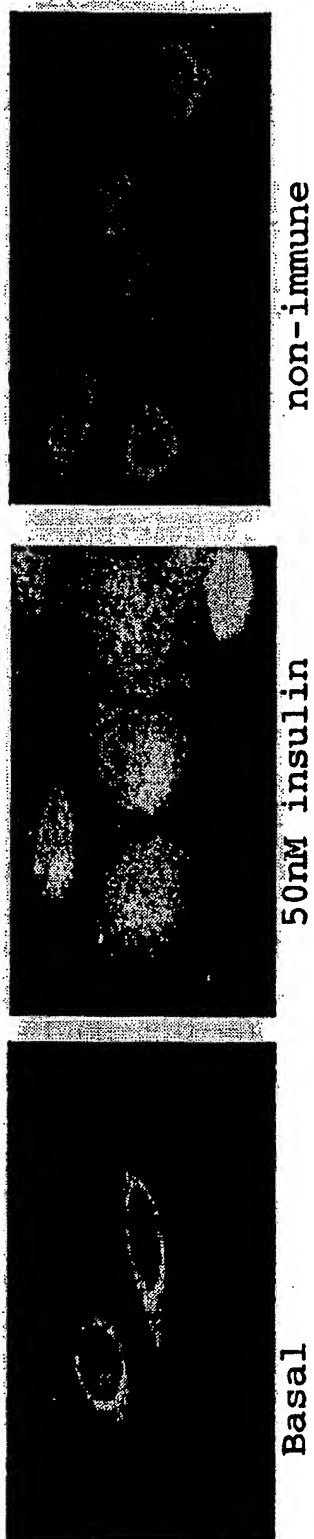


FIGURE 19

MCF7 Northern

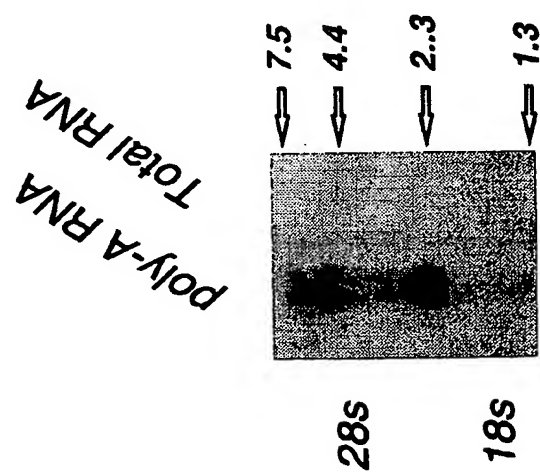


FIGURE 20

- 1 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT:

(A) NAME: St Vincent's Institute of Medical
Research

(B) STREET: 42 Victoria Parade

(C) CITY: Fitzroy

10 (D) STATE: Victoria

(E) COUNTRY: Australia

(F) POSTAL CODE (ZIP): 3065

15 (ii) TITLE OF INVENTION: Transporter Protein

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

20 (B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version
#1.30 (EPO)

25 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

35

(iii) HYPOTHETICAL: NO

- 2 -

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

5

(A) NAME/KEY: misc_feature

(B) LOCATION:1..23

(D) OTHER INFORMATION:/function= "Forward primer"
/product= "Glucose Transport (GLUT4)"

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTTGAGATTG GCCCTGGCCC CAT 23

(2) INFORMATION FOR SEQ ID NO: 2:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

20

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

25

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

30

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION:1..21

(D) OTHER INFORMATION:/function= "Reverse primer"
/product= "Glucose Transport (GLUT4)"

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

- 3 -

GTC(AG)TTCTC ATCTGGCCCT AA 21

(2) INFORMATION FOR SEQ ID NO: 3:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

10

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

15

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

20

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION:1..23

(D) OTHER INFORMATION:/function= "Forward primer"

/product= "Glucose Transport Protein

25

(GLUT8) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTTGAGATTG GNCC(TAC)GGC CC(CG)AT 23

30

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2471 base pairs

35

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- 4 -

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

5

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

10

```
TGTCCCTAAA ATCATCTCAG TACTTGGCAC ACTGACTTAA GATGTGGGGT GGGGGAGCAT 60
CCCTTAACAC ATTCTTTGTT TTCCTGGTAA ATACTGGTGG AACAAAGACAG CTGAGAATGT 120
ATGACATCTG ACCATGAACA TATGACAGCT GTTTGTGCCA GTCATGTCCA AACCCATGGC 180
TCTCAACTCC AGATCCAAAA ACTCTCCCA TGTTTATAGAC CTCCACACC AGCATTTAGG 240
15 ATTTCTTCCT CTATAATCTT GCTGGGTGCT GGTCTTGACG GGCCATCTAC TGGGGATAGG 300
TGGTTTGGGG TCTCAGTGGT GGGCACCGGC TTGTTCTTGC CTCCTCTGCA GCTCCTCTTG 360
CCGCCTCGCC TGCTGTTTAC TCATGCAATC CTTGAACGCC TGCACCTGTG GCTGGCATTG 420
CCGCCAGTCC TGGTGCTGGG CCATGCACTC CTGCACTGCA AAGTGGGAGG CAGCACAGCC 480
AGAGCGGGAG ATCAGCTGGT CCAGCGGGTC CTCCTCCTCA TCGTCTTTCT TCACCGTTGG 540
20 GTCCAGGTAT GGCTTGAGG GACTGAGGTT GACATCCTGG GGATGGGGAG TCGAACAGGT 600
GGGAGAAGAG GGCCGGAAGT CCGGCCGGA TTCCGGATCA GCATTCTTTC TGCTTATATT 660
TCAAATTACG CATTTGCCAA TGTTTCCAT GGCTGGAAGT ACATGTTTGG TCTGTGATT 720
CCCTTGGGAG TTTTGCAAGC AATTGCAATG TATTTCTTTC CTCCAAGCCC TCGGTTTCTG 780
GTGATGAAAG GACAAGAGGG AGCTGCTAGC AAGGTTCTTG GAAGGTTAAG AGCACTCTCA 840
25 GATACACTG AGGAAGTCA TGTGATCAAA TCCTCCCTGA AAGATGAATA TCAGTACAGT 900
TTTTGGGATC TGTTTCGTTT AAAAGACAAC ATGCGGACCC GAATAATGAT AGGACTAACA 960
CTAGTATTTT TTGTACAAAT CACTGGCCAA CAAACATAT TGTTCTATGC ATCAACTGTT 1020
TTGAAGTCAG TTGGATTTC AAGCAATGAG GCAGCTAGCC TCGCCTCCAC TGGGGTTGGA 1080
GTCGTCAAGG TCATTAGCAC CATACTGCC ACTCTTCTTG TAGACCATGT CGGCAGCAAA 1140
30 ACATTCCTCT GCATTGGCTT GCTAAATGCT GGATTAAGCC AACTGAATA CCAGATAGTC 1200
ACAGACCTTG GGGACGTCCC AGCTTTTGTG AAATGGCTGT CCTTAGCCAG CTTGCTTGTT 1260
TATGTTGCTG CTTTTTCAAT TGGTCTAGGA CCAATGCCCT GGCTGGTGCT CAGCGAGATC 1320
TTTCTTGGTG GGATCAGAGG ACGAGCCATG GCTTTAACTT CTAGCATGAA CTGGGGCATC 1380
AATCTCCTCA TCTCGCTGAC ATTTTGTACT GTAAATCTTA TTGGCCTGCC ATGGGTGTGC 1440
35 TTTATATATA CAATCATGAG TCTAGCATCC CTGCTTTTGT TTGTTATGTT TATACCTGAG 1500
ACAAAGGGAT GCTCTTTGGA ACAAATATCA ATGGAGCTAG CAAAAGTGAA CTATGTGAAA 1560
AACAAACATTT GTTTTATGAG TCATCACCAA GAAGAATTAG TGCCAAAACA GCCTCAAAAA 1620
```

- 5 -

AGAAAACCCC AGGAGCAGCT CTTGGAGTGT AACAACTGT GTGGTAGGG CCAATCCAGG 1680
CAGCTTTCTC CAGAGACCTA ATGGCCTCAA CACCTTCTGA ACGTTGGATA GTGCCAGAAC 1740
ACTTAGGAGG GTGNACCTAA TGGCCTCAAC ACCTTCTGAA CGTGGATAGT GCCAGAACAC 1800
TTAGGAGGGT GTCTTTGGAC CAATGCATAG TTGCGACTCC TGTGCTCTCT TTTCAGTGTC 1860
5 CATGGAAGTG GTTTTGAAAA AACACTCCTG AAATTGATAA AANCAGCCTT TAACCCCCCT 1920
CCCTCCCCCA GAAAGGAACC CCCNCAAAGG TTTANNTGAA NGTNACAAAG GTCCCCCTAA 1980
GTTTGATTCT CCCTTTTTTT CCCTNGAAGC CAAGGAATTT ATCCCNNGGT TTTTAAAAA 2040
AAAAAAAAAA AAAAGTTTAA NCCNTGGGCT TGGGGTTTTT TAAATAACTT TTCCCTAACC 2100
CTTCCCTTC CCCACNAGA NATCCCANNC CCCCCCNTT GGAAAATANA AACCTAATGN 2160
10 TTTCCCCCCC TNAAATTTGG AAAAAANAA TTCNAACCCC TTCCCNNGN NNCCCTTTA 2220
AAGCCCCTAA TGGTTTAAAT GGTTTTAAAT NGGGGAANGG GCCCAANTTT CCCCCCCAA 2280
GCCTTTTTTT ATTTAATGG CCNAANAACC NCCCCCAAG TTTNGGGTCC CCCTGGGGAA 2340
ACAATGAANG GGGTTTTTNC NNTTTTCCCC TTGGGCCCT TACCCAAAA AAANACTTAA 2400
CCTTTGGCAC CNGGNAAT TTCTTTTAAA CCCCCAAAA AAAAAACNC CNTAAAACCC 2460
15 CCCCTTTTTT C 2471

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 526 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(vi) ORIGINAL SOURCE:

- 30 (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: Protein
(B) LOCATION: 1..526
35 (D) OTHER INFORMATION: /note= "Deduced amino acid
sequence of GLUT8"

- 6 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

	Met	Thr	Ser	Asp	His	Glu	His	Met	Thr	Ala	Val	Cys	Ala	Ser	His	Val
	1				5					10					15	
5	Gln	Thr	His	Gly	Ser	Gln	Leu	Gln	Ile	Gln	Lys	Leu	Ser	Pro	Cys	Phe
				20				25					30			
	Arg	Pro	Pro	Thr	Pro	Ala	Phe	Arg	Ile	Ser	Ser	Ser	Ile	Ile	Leu	Leu
				35				40					45			
	Gly	Ala	Gly	Leu	Ala	Gly	Pro	Ser	Thr	Gly	Asp	Arg	Trp	Phe	Gly	Val
10		50					55					60				
	Ser	Val	Val	Gly	Thr	Gly	Leu	Phe	Leu	Pro	Pro	Leu	Gln	Leu	Leu	Leu
	65					70				75					80	
	Pro	Pro	Arg	Leu	Leu	Phe	Thr	His	Ala	Ile	Leu	Glu	Arg	Leu	His	Leu
					85					90					95	
15	Trp	Leu	Ala	Leu	Pro	Pro	Val	Leu	Val	Leu	Gly	His	Ala	Leu	Leu	His
				100				105						110		
	Cys	Lys	Val	Gly	Gly	Ser	Thr	Ala	Arg	Ala	Gly	Asp	Gln	Leu	Val	Gln
				115				120					125			
	Arg	Val	Leu	Leu	Leu	Ile	Val	Phe	Leu	His	Arg	Trp	Val	Gln	Val	Trp
20		130					135					140				
	Pro	Glx	Gly	Thr	Glu	Val	Asp	Ile	Leu	Gly	Met	Gly	Ser	Arg	Thr	Gly
	145					150				155					160	
	Gly	Arg	Arg	Gly	Pro	Glu	Leu	Arg	Pro	Gly	Phe	Arg	Ile	Ser	Ile	Leu
					165					170					175	
25	Ser	Ala	Tyr	Ile	Ser	Asn	Tyr	Ala	Phe	Ala	Asn	Val	Phe	His	Gly	Trp
				180					185						190	
	Lys	Tyr	Met	Phe	Gly	Leu	Val	Ile	Pro	Leu	Gly	Val	Leu	Gln	Ala	Ile
				195				200					205			
	Ala	Met	Tyr	Phe	Leu	Pro	Pro	Ser	Pro	Arg	Phe	Leu	Val	Met	Lys	Gly
30		210					215					220				
	Gln	Glu	Gly	Ala	Ala	Ser	Lys	Val	Leu	Gly	Arg	Leu	Arg	Ala	Leu	Ser
	225					230				235					240	
	Asp	Thr	Thr	Glu	Glu	Leu	Thr	Val	Ile	Lys	Ser	Ser	Leu	Lys	Asp	Glu
				245					250					255		
35	Tyr	Gln	Tyr	Ser	Phe	Trp	Asp	Leu	Phe	Arg	Ser	Lys	Asp	Asn	Met	Arg
				260					265					270		
	Thr	Arg	Ile	Met	Ile	Gly	Leu	Thr	Leu	Val	Phe	Phe	Val	Gln	Ile	Thr

- 7 -

	275	280	285
	Gly Gln Pro Asn Ile Leu Phe Tyr Ala Ser Thr Val Leu Lys Ser Val		
	290	295	300
	Gly Phe Gln Ser Asn Glu Ala Ala Ser Leu Ala Ser Thr Gly Val Gly		
5	305	310	315 320
	Val Val Lys Val Ile Ser Thr Ile Pro Ala Thr Leu Leu Val Asp His		
	325	330	335
	Val Gly Ser Lys Thr Phe Leu Cys Ile Gly Leu Leu Asn Ala Gly Leu		
	340	345	350
10	Ser His Thr Glu Tyr Gln Ile Val Thr Asp Pro Gly Asp Val Pro Ala		
	355	360	365
	Phe Leu Lys Trp Leu Ser Leu Ala Ser Leu Leu Val Tyr Val Ala Ala		
	370	375	380
	Phe Ser Ile Gly Leu Gly Pro Met Pro Trp Leu Val Leu Ser Glu Ile		
15	385	390	395 400
	Phe Pro Gly Gly Ile Arg Gly Arg Ala Met Ala Leu Thr Ser Ser Met		
	405	410	415
	Asn Trp Gly Ile Asn Leu Leu Ile Ser Leu Thr Phe Leu Thr Val Asn		
	420	425	430
20	Leu Ile Gly Leu Pro Trp Val Cys Phe Ile Tyr Thr Ile Met Ser Leu		
	435	440	445
	Ala Ser Leu Leu Phe Val Val Met Phe Ile Pro Glu Thr Lys Gly Cys		
	450	455	460
	Ser Leu Glu Gln Ile Ser Met Glu Leu Ala Lys Val Asn Tyr Val Lys		
25	465	470	475 480
	Asn Asn Ile Cys Phe Met Ser His His Gln Glu Glu Leu Val Pro Lys		
	485	490	495
	Gln Pro Gln Lys Arg Lys Pro Gln Glu Gln Leu Leu Glu Cys Asn Lys		
	500	505	510
30	Leu Cys Gly Arg Gly Gln Ser Arg Gln Leu Ser Pro Glu Thr		
	515	520	525

(2) INFORMATION FOR SEQ ID NO: 6:

- 35 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid

- 8 -

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
5 (A) DESCRIPTION: /desc = "PCR primer"

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
10 (A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..19
15 (D) OTHER INFORMATION: /function= "Forward primer"
/product= "Glucose transport protein
(GLUT8)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
20
TCCATGGCTG GAAGTACAT 19

(2) INFORMATION FOR SEQ ID NO: 7:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(iii) HYPOTHETICAL: NO
35

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

- 9 -

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION:1..19

5 (D) OTHER INFORMATION:/function= "Reverse primer"
/product= "Glucose transport protein
(GLUT8)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

10

TAAGTGTTCCT GGCACATATC 19

(2) INFORMATION FOR SEQ ID NO: 8:

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

(iii) HYPOTHETICAL: NO

25

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

30

(A) NAME/KEY: misc_feature

(B) LOCATION:1..18

(D) OTHER INFORMATION:/function= "Forward primer"
/product= "Glucose transport protein
(GLUT8)"

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

- 10 -

TCAACATCCA CATGAACT 18

(2) INFORMATION FOR SEQ ID NO: 9:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"
- (iii) HYPOTHETICAL: NO
- 15 (vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
- (ix) FEATURE:
20 (A) NAME/KEY: misc_feature
(B) LOCATION: 1..21
(D) OTHER INFORMATION: /function= "Reverse primer"
/product= "Glucose transport protein
(GLUT8)"
- 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TGAAAAAGCA GCAACATAAA C 21

30 (2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
35 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- 11 -

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

(iii) HYPOTHETICAL: NO

5

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

10

(A) NAME/KEY: misc_feature

(B) LOCATION: 1..18

(D) OTHER INFORMATION: /function= "Forward primer"

/product= "Non-oestrogen-dependent

house-keeping gene-36B4"

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TGGGCTCCAA GCAGATGC 18

20 (2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

25

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

30

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapien

35

(ix) FEATURE:

(A) NAME/KEY: misc_feature

- 12 -

(B) LOCATION:1..18

(D) OTHER INFORMATION:/function= "Reverse primer"
/product= "Non-oestrogen-dependent
house-keeping gene-36B4"

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGCTTCGCTG GCTCCCAC 18

10 (2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

15 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

20 (iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:

25 (A) ORGANISM: Homo sapiens

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION:1..16

30 (D) OTHER INFORMATION:/note= "C-terminus of GLUT8
used to generate polyclonal antibody"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

35 Asn Lys Leu Cys Gly Arg Gly Gln Ser Arg Gln Leu Ser Pro Glu Thr
1 5 10 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 98/00819

A. CLASSIFICATION OF SUBJECT MATTER												
Int Cl ⁶ : C07K 14/47, 7/08; C12N 15/12, C12Q 1/68; G01N 33/53, 33/577, 33/68												
According to International Patent Classification (IPC) or to both national classification and IPC												
B. FIELDS SEARCHED												
Minimum documentation searched (classification system followed by classification symbols)												
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched												
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) ANGIS CAS ONLINE MEDLINE												
C. DOCUMENTS CONSIDERED TO BE RELEVANT												
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.										
X A	Proc. Natl. Acad. Sci. USA, Vol. 90, published December 1993, pages 11658-11662 J. Fischbarg et al, "Evidence that facilitative glucose transporters may fold as β -barrels" Page 11658 column 2 Whole document	17-19 1-31										
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input type="checkbox"/> See patent family annex												
<p>* Special categories of cited documents:</p> <table border="0"> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td>"&" document member of the same patent family</td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	"P" document published prior to the international filing date but later than the priority date claimed	
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"P" document published prior to the international filing date but later than the priority date claimed												
Date of the actual completion of the international search 5 November 1998		Date of mailing of the international search report 12 NOV 1998										
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929		Authorized officer <i>Chait</i> OI LEE CHAI Telephone No.: (02) 6283 2482										

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 98/00819

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	Proc. Natl. Acad. Sci. USA, Vol. 88, published August 1991, pages 6893-6897, R.M. Smith et al, "Immunoelectron microscopic demonstration of insulin-stimulated translocation of glucose transporters to the plasma membrane of isolated rat adipocytes and masking of the carboxyl-terminal epitope of intracellular GLUT4" Page 6894 column 2 Whole document	17-19 1-31
A	Diabetes, Vol. 41, published November 1992, pages 1436-1445, J.B. Buse et al "Human GLUT4/Muscle-Fat Glucose-Transporter Gene-Characterisation and Genetic Variation" Whole document	1-31
A	J. Anim. Sci, Vol. 75, published January 1997, pages 182-188, H. Abe et al, "Molecular Cloning and mRNA expression of the bovine insulin-responsive glucose transporter (GLUT4)" Whole document	1-31
P, A	The Journal of Veterinary Medical Science, Vol. 60, published June 1998, pages 769-771, H. Abe et al, "Comparison of Amino Acid Sequence of the C-Terminal Domain of Insulin-Responsive Glucose Transporter (GLUT4) in Livestock Mammals" Whole document	1-31